University of Alberta

PROTEIN QUALITY CONTROL IN THE CALRETICULIN DEFICIENT ENDOPLASMIC RETICULUM

by

IRFAN AHSAN

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

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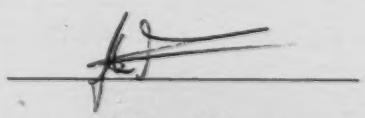

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ABSTRACT

Quality control in the endoplasmic reticulum (ER) relies on the interaction of newly synthesized proteins with chaperones and folding factors found in the ER. Although defective protein folding leads to severe pathologies, limited information is available concerning the molecular events that govern the folding of proteins in the ER lumen. Calnexin and calreticulin are molecular chaperones which are involved in the folding, assembly, and retention of proteins. Calreticulin-deficiency is embryonic lethal, but the contribution of chaperone function to this phenotype is not known. Here I investigated how calreticulin deficiency in the lumen of the ER affects protein folding. I found that the expression of several important ER chaperones is changed in the absence if calreticulin. I also show that protein folding is accelerated in the absence of calreticulin, and that quality control is compromised. There is also an accumulation of unfolded proteins and a triggering of the unfolded protein response. My work indicates that the absence of one chaperone in the ER can compromise overall protein quality control.

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LIST OF ABREVIATIONS

ALLN	N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal
Asn	asparagine
Asp	Aspartic acid
ATP	adenosine triphosphate
Ca^{2+}	calcium ion
CFTR	cystic fibrosis transmembrane conductance regulator
CNX	calnexin
CRT	calreticulin
crt-/-	calreticulin deficient
CST	castanospermine
Da	Dalton
DMEM	Dulbecco's Modified Eagles Medium
ER	endoplasmic reticulum
FBS	fetal bovine serum
Glu	Glutamic acid
HBS	Hepes buffered saline
IP_3	inositol triphosphate
kDa	kilo Dalton
Leu	Leucine
Lys	Lysine
MEF	mouse embryonic fibroblasts

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDI	protein disulfide isomerase
RyR	ryanodine receptor
SDS	sodium dodecyl sulfate
Ser	serine
SERCA	sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase
TBS	tris buffered saline
Thr	threonine
TUN	tunicamycin
UGGT	UDP-glucose:glycoprotein glucosyltransferase
UPR	unfolded protein response
WT	wildtype

CHAPTER ONE

INTRODUCTION

General Introduction

Proteins are essential to all known biological processes, for they perform or are involved in virtually every process, function, and component of the living cell. However, for proteins to perform their correct functions, each must first fold into a distinct three dimensional arrangement that characterizes the protein and allows it to perform its function, and remain in this conformation for a biologically relevant period of time. According to a concept known as Anfinsen's dogma (Anfinsen et al., 1961), the characteristic fold of each protein is determined by its amino acid sequence. However, given the number of positions each amino acid in a polypeptide chain can adopt, the total number of possible conformations available for a protein sequence to fold is enormous. If a protein sampled all its possible arrangements, its folding would be too slow for biological requirements. This concept is known as Levinthal's paradox (Levinthal, 1968). Proteins, however, can reproducibly fold on a time scale compatible with biological processes, thus *in vivo* there must clearly be folding factors which assist and speed up the folding process. Indeed there are many molecular chaperones in the cytosol of the cell and its other compartments, which help stabilize unfolded structures of polypeptides thus preventing their non-functional aggregation and ensuring that the folding of proteins occurs both rapidly and efficiently so that proteins reach their correct, functional conformation.

Protein folding in the endoplasmic reticulum

The endoplasmic reticulum (ER) is the most abundant membranous organelle found in eukaryotic cells. The ER plays a central role in many cellular functions. It is the site of synthesis and transport of membrane proteins and lipids. It is also the major Ca^{2+} storage facility in the cell. In addition to these functions, the ER is the compartment of biosynthesis and maturation of proteins and glycoproteins that are destined for secretion, the plasma membrane, and for transport to various other cellular organelles. Approximately one-third of all cellular proteins are translocated into the lumen of the ER on membrane-bound ribosomes, where post-translational modification, folding, and oligomerization occurs (Kaufman, 1999; Mori, 2000). For proteins that are destined to leave the cell or reside at the plasma membrane, trafficking occurs to the Golgi compartment and then to the cell surface via membrane vesicles. This is called the secretory pathway.

Due to the nature of secreted proteins and the nature of the ER lumenal environment, protein folding in the ER presents a challenge. The ER, however, is unique in that it provides an oxidizing environment in which numerous protein chaperones reside that facilitate and promote the productive folding of proteins and protein complexes despite the presence of high concentrations of protein that would otherwise interfere with productive folding reactions (Kaufman, 1999). Proteins of the secretory pathway are generally rich in cysteine residues and

acquire multiple disulfide bonds. The orderly formation of these bonds is necessary for both the stability of the final product and also for the folding progress of newly synthesized proteins. The ER lumen has ideal redox conditions for this purpose. The main redox buffer in the ER is provided by glutathione. In contrast to the ratio of reduced to oxidized glutathione of 100:1 in the cytosol, the ratio is 10:1 in the lumen of the ER, which experiments show is an optimal ratio for spontaneous disulphide bond formation *in vitro* (Helenius et al., 1992). Within the lumen of the ER, the concentration of protein is estimated to reach 100 mg/mL (Stevens and Argon, 1999). These conditions are conducive to aggregation, especially for newly synthesized polypeptide chains as they have many exposed hydrophobic regions which would normally be buried in the native structure. The likelihood of co-aggregation of nascent chains is also very high since these polypeptides must fold simultaneously in the presence of thousands of different proteins. However, the ER contains a variety of proteins that prevent aggregation and ensure that nascent polypeptides fold properly (Gething and Sambrook, 1992).

Being the major folding compartment for secreted and membrane proteins, the ER contains many folding factors and enzymes that assist and speed up the folding of newly synthesized proteins. These protein chaperones provide two important functions in protein folding. First, the enzymatic proteins such as protein disulfide isomerase and *cis-trans* prolyl isomerase catalyze protein-folding reactions and increase the rate at which proteins fold. Second, there are protein

chaperones that do not actively catalyze protein folding but assist it by maintaining unfolded proteins in a folding competent state. These proteins prevent unfolded proteins from aggregating and stabilize unfavourable conformations of folding intermediates (Stevens and Argon, 1999).

Given the complex demands of folding of secreted and membrane proteins, the ER contains many types of protein chaperones, several of which are members of the main families of molecular chaperones. The primary peptide binding chaperone is BiP, a member of the HSP70 family of chaperones. BiP was first identified as the immunoglobulin heavy chain binding protein (Haas and Wabl, 1983), however its function as a broad specificity molecular chaperone was realized when it was shown that BiP is ER located and a member of the HSP70 family of molecular chaperones (Munro and Pelham, 1986). BiP assists in the assembly and folding of newly synthesized proteins by recognizing and binding unfolded polypeptides, and thus inhibiting intra- and intermolecular aggregation. By doing so, BiP is able to maintain unfolded proteins in a folding competent state. BiP requires ATP for its function as do many ER chaperones, and ATP binding to and hydrolysis by BiP allows binding and release of peptides during the folding process (Gething, 1999). The role of BiP as a general chaperone in the ER lumen is due to its ability to recognize and bind a wide variety of nascent polypeptides that have no particular sequence similarity. In general, these polypeptide regions are highly hydrophobic, indicating that BiP interacts with sequences normally located in the interior of a fully folded protein. BiP is an

important ER protein in that it not only functions as a molecular chaperone, but is also involved in the signalling pathway of the ER unfolded protein response which will be discussed later.

The only HSP90 family of chaperone found in the ER is GRP94. GRP94 is among the most abundant of ER proteins and is a major calcium binding protein of the ER (Koch et al., 1986). It is known to act as a molecular chaperone by binding peptide sequences, but apparently these are a different subset than that of BiP. GRP94 is probably less of a global chaperone than BiP as it has been only shown to interact with a restricted set of proteins. The expression of GRP94 is up-regulated in response to a number of ER stress conditions which lead to the unfolded protein response (Argon and Simen, 1999). A feature common to these stress conditions is that they lead to the accumulation of misfolded proteins in the ER (Kaufman, 1999).

The presence of disulfide bonds is generally a distinguishing feature of proteins that fold in the ER as opposed to those that fold in the cytosol (Stevens and Argon, 1999). Since the ER is an oxidizing environment, some cysteine residues in newly synthesized polypeptides tend to oxidize as they appear in the lumen. However, there are clearly enzymes in the ER that delay the oxidation of cysteine residues until a degree of protein folding has already occurred (Stevens and Argon, 1999). This may be due to the necessity of certain regions requiring to be folded before disulfide bond formation, which would indicate that disulfide

bonds serve to stabilize a local area in the folded structure. Nevertheless, this requires a precise control of protein oxidation in the ER. For this purpose, the protein thiol oxidoreductase family of proteins exist of which the most abundant in the ER include protein disulfide isomerase (PDI) (Freedman, 1989), and two structurally related proteins ERp57 (Lewis et al, 1986), and ERp72 (Lewis et al, 1985). These enzymes are responsible for catalyzing the oxidation of cysteine residues to form disulfide bonds, and the isomerization of incorrectly paired disulfides on nascent polypeptide chains undergoing folding in the oxidizing environment of the ER. Indeed these molecular chaperones interact directly with their substrates as they have been shown to form mixed disulfides with their substrates *in vivo* (Molinari and Helenius, 1999).

The molecular chaperone composition of the ER differs from that of other folding compartments in the cell in that it contains a unique type of chaperone. These chaperones are lectins that bind glycoproteins by their glycan moieties. The two known such chaperones are calreticulin, a lumenal protein, and calnexin, the only membrane spanning chaperone identified so far (Chevet et al., 1999; Stevens and Argon, 1999). The specificity of both calreticulin and calnexin is identical -- they bind to monoglucosylated intermediates of glycan trimming (Helenius et al., 1997; Ware et al., 1995). There is also evidence however that they bind to the peptide portions of newly synthesized proteins as well (Ihara et al., 1999; Saito et al., 1999). Calnexin and calreticulin play a distinct and important role in the folding of proteins and glycoproteins in the ER. The binding

properties and the role in protein folding played by calnexin and calreticulin are discussed in more detail below. A summary of the ER chaperones and folding enzymes discussed in this section is shown in Table 1-1.

The calreticulin/calnexin cycle

One of the unique properties of the proteins that fold inside the ER is that many of them are glycosylated, i.e. they are covalently modified by glycan moieties. These are usually secreted proteins or proteins that will reside at the surface of the plasma membrane. In eukaryotic cells, N-linked glycans serve a variety of functions. Of the most important of these functions is the promotion of correct folding of newly synthesized polypeptides in the ER (Helenius and Aebi, 2001). This unique eukaryotic adaptation allows cells to produce and secrete large, complex proteins at high levels. Inhibition of glycosylation generally results in the accumulation of misfolded, aggregated proteins that are dysfunctional.

The synthesis of N-linked glycans in eukaryotic cells begins by the assembly of a 14-saccharide unit as a membrane bound dolichylpyrophosphate precursor by enzymes on both sides of the ER membrane (Gahmberg and Tolvanen, 1996; Helenius and Aebi, 2001). Synthesis starts on the cytosolic side of the ER membrane by the addition of two N-acetylglucosamines and five mannose residues to dolichylphosphate. At this stage, the oligosaccharide is

flipped to the lumenal side of the membrane and four additional mannose residues and three glucose residues are added on to the chain. Once this is complete, the oligosaccharyltransferase enzyme complex transfers the core oligosaccharide unit onto asparagine residues of a growing polypeptide chain (Silberstein and Gilmore, 1996). The oligosaccharide unit is added to the asparagine residues of the glycosylation sequence motif consisting of Asn-X-Ser/Thr (Bause, 1983). The three terminal glucose residues can then be trimmed by glucosidases I (Kalz-Fuller et al, 1995) and II (Ray et al, 1991) and the terminal mannoses by several ER mannosidases. The ER also contains a glucosyltransferase, which will be discussed later, that can reglucosylate glucose-free chains and thus establish, with glucosidase II, a deglucosylation/re-glucosylation cycle (see Figure 1-1 for oligosaccharide biosynthesis). When the glycoprotein has folded and reached the Golgi complex, further sugar trimming occurs for various purposes. The glycoforms generated in the ER are homogenous, however the Golgi-generated forms are highly diverse and vary between species (Helenius and Aebi, 2001; Helenius et al., 1997).

The most important effect of glycans on the folding process involves a unique chaperone system found in the ER called the calreticulin/calnexin cycle (Helenius and Aebi, 2001). The glycans serve to target glycoproteins to this cycle. Calreticulin and calnexin are lectins that will bind to almost all newly synthesized glycoproteins via the glycan moieties after they have been trimmed by glucosidases I and II to the monoglucosylated form ($Glc_1Man_{9-6}GlcNAc_2$).

During this interaction period, folding of the polypeptide occurs while associated with either calnexin or calreticulin. Dissociation of the glycopeptide from the chaperone occurs when the terminal glucose residue of the glycan is removed by glucosidase II. If, however, the glycoprotein is incorrectly or incompletely folded, the enzyme UPD-glucose:glycoprotein glucosyltransferase (UGGT) (Fernandez et al, 1996; Parker et al, 1995) adds a terminal glucose residue back on to the glycan so that re-association can occur with either calreticulin or calnexin. Thus, this allows a cycle of binding to and dissociation from the lectin chaperone until the glycoprotein is completely folded, whereupon it then leaves this cycle and enters the secretory pathway. In this cycle, UGGT serves as a folding sensor, and studies have shown that this enzyme only reglucosylates incompletely folded glycoproteins (Trombetta and Helenius, 2000). Thus by recognizing the folding status, UGGT forces incompletely folded glycoproteins to remain in the calreticulin/calnexin cycle until they are completely folded and no longer recognized by this enzyme and are then allowed to proceed in the secretory pathway (see Fig. 1-2 for a summary of the calreticulin calnexin cycle).

Quality Control in the endoplasmic reticulum and the Unfolded Protein Response

The efficiency of protein folding in the endoplasmic reticulum varies dramatically depending on the type of protein and is not always quantitative

Figure 1-1. Biosynthesis of the N-linked core oligosaccharide. Synthesis of the glycan moiety starts on the cytosolic surface of the ER membrane with the addition of the sugars to the dolichylphosphate. The oligosaccharide is then flipped to the lumenal side of the membrane and sugar addition is completed and the entire complex is then transferred to the growing nascent polypeptide chain. ER Glucosidases I and II then trim the glucose residues. Once folding is complete, the glycoprotein then moves into the Golgi and into the secretory pathway, or to the targets for which it is destined.

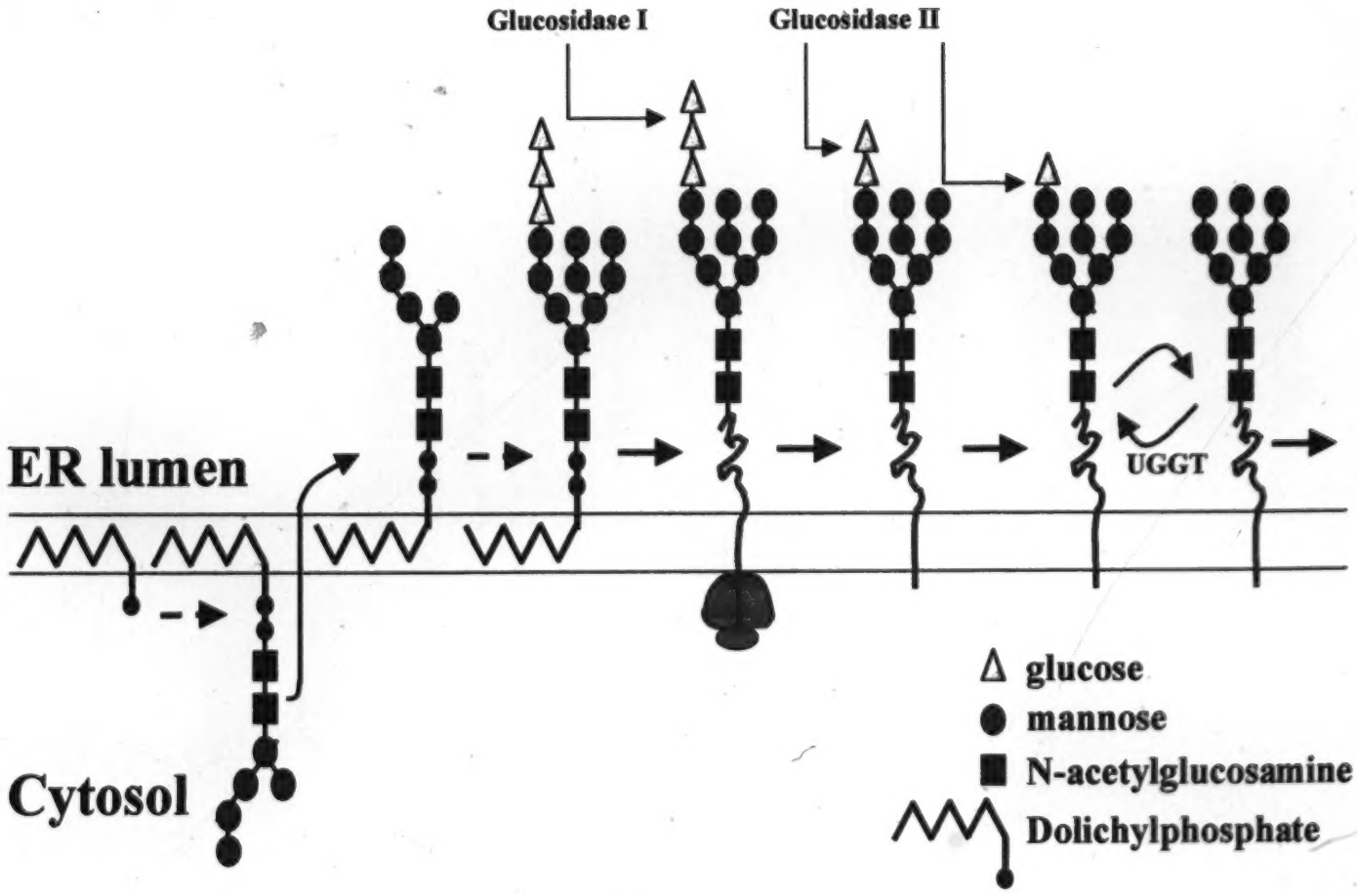

Glucosidase I
Glucosidase II
UGGT
ER lumen
Cytosol
glucose
mannose
N-acetylglucosamine
Dolichylphosphate

Figure 1-1

Figure 1-2. The calreticulin/calnexin chaperone cycle. Newly synthesized glycoproteins are trimmed by glucosidases I and II to the monoglucosylated form of the glycan. This form can interact with both calreticulin and calnexin in cycles of folding and unfolding until the polypeptide is fully folded. The interaction between the glycan and the lectin chaperones is broken by the removal of the terminal glucose residue by glucosidase II. If the protein is not folded, this is sensed by the enzyme UGGT which then reglucosylates the glycan to allow further interaction with calnexin and calreticulin until folding is complete. Once the protein is properly folded it then leaves the cycle and enters the secretory pathway. The entire chaperoning process occurs within the ER lumenal environment. UGGT, UDP-glucose:glycoprotein glucosyltransferase.

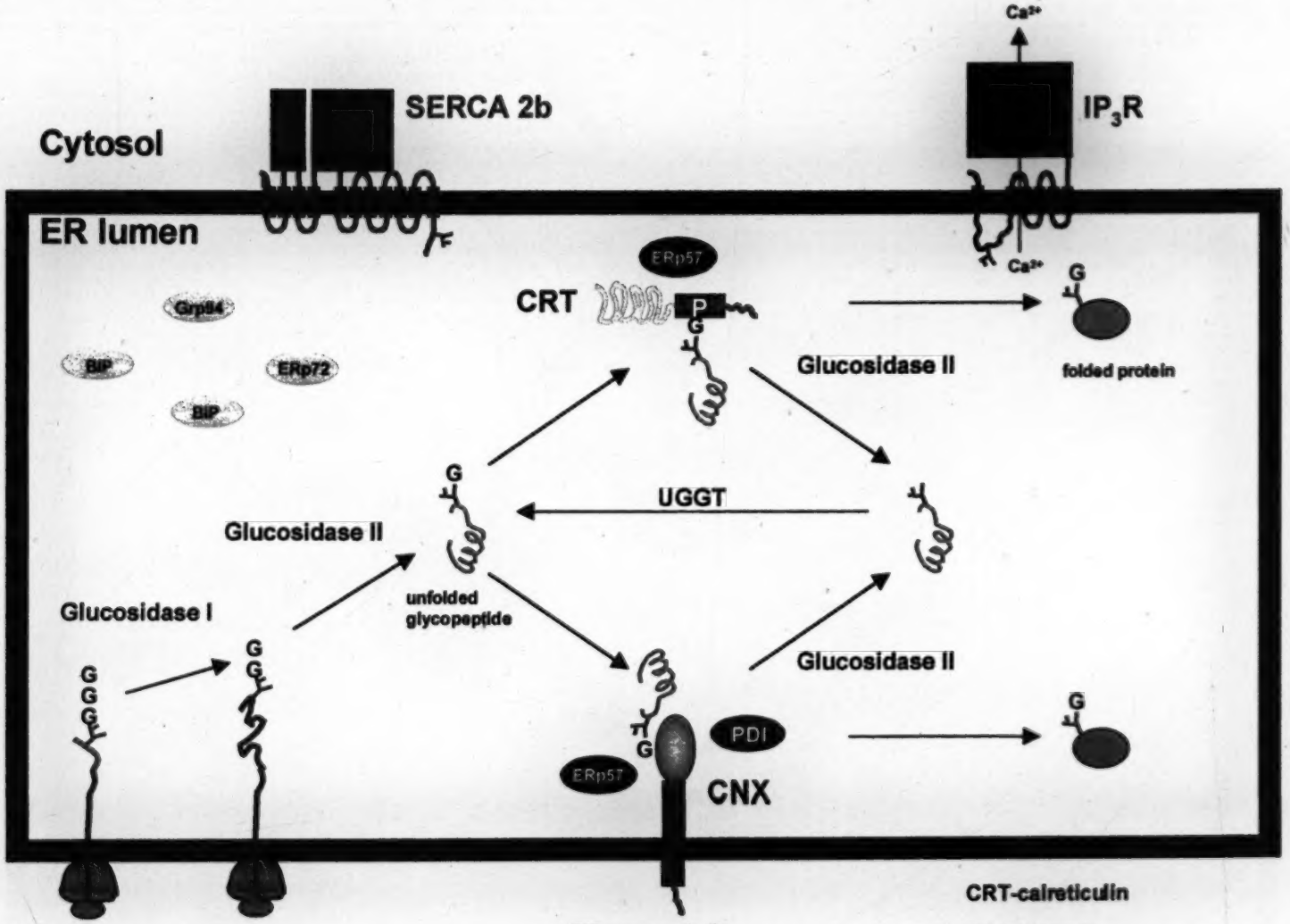

Cytosol
SERCA 2b
IP3R
Ca2+
ER lumen
ERp57
CRT
P
G
Grp94
BiP
ERp72
BiP
Glucosidase II
folded protein
UGGT
Glucosidase II
unfolded
glycopeptide
Glucosidase I
Glucosidase II
PDI
ERp57
CNX
CRT-calreticulin
CNX-calnexin

Figure 1-2

(Stevens and Argon, 1999). Misfolded or incompletely folded proteins are common side products of protein synthesis in the ER, and thus there must exist a mechanism to sort these away from native proteins and ER resident proteins and target them for degradation. For incompletely folded proteins, transport out of the ER must be prevented to ensure the fidelity of the system and to ensure that only functional proteins reach their final destination. The term quality control describes the conformation-dependent molecular sorting of newly synthesized protein in the ER (Ellgaard and Helenius, 2001; Ellgaard et al., 1999). ER quality control operates at several levels, some of the controls apply to all proteins, and some apply to specific proteins and protein families. At the general level, however, all proteins are subject to conformation based molecular sorting by members of the major chaperone families in the ER. The most common mechanism of quality control involves the association of newly synthesized proteins with ER chaperones and folding enzymes such as BiP, calnexin, calreticulin, GRP94, and the thiol oxidoreductases PDI, ERp57, and ERp72. These factors have the ability to recognize properties common to unfolded proteins such as exposed hydrophobic regions, and by selectively associating with such features, they assist in folding and assembly. These interactions also serve as retention anchors for unfolded proteins, and thus the chaperones prevent the forward transport of unfolded proteins out of the ER. The chaperones and folding enzymes themselves remain localized to the ER because they possess specific retention and retrieval signals. Most ER resident proteins contain a C-terminal Lys-Asp-Glu-Leu (KDEL) sequence (Nilsson and Warren, 1994), and type I

membrane proteins have a KKXX (where X may be amino acid) sequence which allows these proteins to remain in the ER via a receptor mediated retrieval mechanism (Andersson et al, 1999; Nilsson and Warren, 1994).

Although general association with chaperones and retention in the ER is the major mechanism of quality control, each of the different chaperones has its unique molecular mechanism of retaining unfolded proteins. BiP and GRP94 for example, bind to exposed peptide regions of unfolded polypeptide chains (Argon and Simen, 1999; Gething, 1999), and calnexin and calreticulin bind through lectin interactions with glycopeptides (Helenius et al., 1997). The calreticulin/calnexin cycle in itself serves as an effective retention-based quality control method. Unfolded glycoproteins cannot escape binding to these chaperones unless deglucosylated by glucosidase II, and if they are still recognized as unfolded by the folding sensor UGGT, they will be reglucosylated and binding to the lectin chaperones will again occur (Trombetta and Helenius, 2000). It is generally thought however, that the chaperone system is quite flexible, where chaperones are able to substitute for each other, cooperate in different ways, and respond differently to physiological changes and cellular stress (Ellgaard and Helenius, 2001).

Another important strategy for quality control in the ER involves a degradation system for misfolded proteins (Brodsky and McCracken, 1999). Proteins, which persistently remain misfolded or unassembled, either become

aggregated or degraded. The process of ER-associated degradation (ERAD) is an important mechanism of quality control in the ER (Brodsky and McCracken, 1999; Plemper and Wolf, 1999). Without the degradation of misfolded proteins, these would accumulate in the ER and cause intracellular problems, and in fact many human diseases are related to the improper folding of specific molecules. In general, the process of ERAD occurs in several steps. Terminally misfolded proteins are recognized by ER chaperones such as calnexin, BiP, and others. They are then retrotranslocated through the Sec61 channel into the cytosol (Plemper and Wolf, 1999), and targeted for degradation by the addition of a polyubiquitin tag. Proteins tagged in this manner are recognized and degraded by the 26S proteasome located in the cytosol (Brodsky and McCracken, 1999; Ellgaard et al., 1999; Plemper and Wolf, 1999). The exact mechanism for the recognition of unfolded proteins to be degraded is still unclear, however in the case of glycoproteins, a Man_8 form of the glycan chain is thought to be responsible for targeting the glycopeptide for proteasome mediated degradation (Helenius and Aebi, 2001; Liu et al., 1999).

The accumulation of misfolded proteins is potentially devastating to the cell, as this would affect the function and/or localization of nearly one third of all cellular proteins, the fraction that is translocated and folded in the ER (Kaufman, 1999; Mori, 2000). When protein misfolding occurs in the ER, the cell initiates a number of processes as part of the quality control system that the ER utilizes to maintain a flow of correctly folded proteins. In eukaryotic cells, the accumulation

of misfolded polypeptides in the ER lumen results in the transcriptional up-regulation of many of the genes encoding ER resident chaperones and folding enzymes (Gething, 1999; Kaufman, 1999; Mori, 2000). This unique intracellular signalling pathway is known as the unfolded protein response (UPR) and connects the ER with the nucleus. The UPR is a combination of three separate mechanisms the cell uses to deal with the situation caused by increased levels of misfolded proteins – activation of transcription of chaperone genes, attenuation of general protein translation, and increase in protein degradation in the ER (Mori, 2000). Cells respond to an increased level of unfolded proteins in the ER lumen by activating transcription of a set of genes involved in protein folding, ex. BiP, GRP94, calreticulin, and PDI. The UPR can therefore adjust the protein folding capacity of the ER according to need, thus alleviating the increased demand on the existing cellular protein folding machinery. In addition to this, general protein translation is decreased, through phosphorylation of the transcription factor eukaryotic translation initiation factor 2α (eIF-2α). Signalling occurs through two ER trans-membrane proteins, Inositol response element 1 protein (Ire1) (Wang et al, 1998; Tirasophon et al, 1998), and PKR-like ER kinase (PERK; PKR, interferon-inducible RNA dependent protein kinase) (Harding et al., 1999). The lumenal domains of Ire1 and PERK recognize ER stress, while their cytosolic domains are involved in the specific cellular response that occurs. Recent studies have shown that the lumenal domain of the two proteins are functionally interchangeable, and have found that in unstressed cells, both domains form stable complexes with the ER chaperone BiP (Bertolotti et al., 2000). Perturbation of

protein folding promoted the dissociation of BiP, allowing activation of both Ire1 and PERK. These findings are consistent with a model in which binding of BiP represses signalling through PERK or Ire1. Protein misfolding relieves this repression by causing the release of BiP from PERK and Ire1 lumenal domains, thus allowing their activation. Thus BiP, along with its chaperone activity, is an important effector of the UPR pathway and a marker for ER stress due to increased misfolded proteins as it is drastically increased during the UPR response (Gething, 1999).

Defects in protein folding and human disease

Many human diseases arise from mutations in proteins that affect their ability to fold into their native structure. These so called conformational diseases include many known common human diseases such as Alzheimer's and Parkinson's diseases, amyloidoses, and α_1-antitrypsin deficiency (Chevet et al., 1999; Kopito and Ron, 2000). These arise when a specific protein undergoes a conformational change resulting in it acquiring a tendency to aggregate and accumulate within tissues or cellular compartments. It is the accumulation of these abnormal proteins and the resulting cytotoxicity that causes the disease state. One of the best known examples of conformational disease is α_1-antitrypsin (α_1-AT) deficiency. α_1-AT is an abundant serum glycoprotein secreted by the liver, the normal function of which is to bind and inactivate elastase which is a

protease produced by neutrophils. The resulting loss of function phenotype is lung damage caused by the destruction of the lung parenchyma from uncontrolled elastase activity. The hepatotoxic effect of the accumulated abnormally folded α_1-AT molecules in the ER lumen is also a leading cause of liver disease in children. Alzheimer's disease is an inherited amyloidosis disease, which occurs due to the production of unique conformational intermediates of the amyloid β–protein precursor (βAPP). The genetic defect initiating the of onset this disease lies in ER processing pathways. Aberrant proteolytic processing of the transmembrane βAPP glycoprotein results in the production of a peptide product that then accumulates and polymerizes in the ER lumen and cytosol, resulting in the production of senile plaques causing neurodegeneration (Aridor and Balch, 1999). Cystic fibrosis is a genetic disorder caused by the absence of a plasma membrane chloride channel. The most common mutant allele causing cystic fibrosis is the deletion of phenylalanine 508 (ΔF508) in the cystic fibrosis transmembrane conductance regulator (CFTR) (Welsh and Smith, 1993). This specific mutation in this chloride ion channel interferes with the correct folding of the protein and affects the conformation preventing it from being transported to the cell surface where it normally functions, and the mutant form remains in the ER and is eventually degraded. These are only a few examples of conformational diseases and there are many more which exist, for example Creutzfeld-Jacob syndrome, Parkinson's disease, and familial hypercholesterolemia, and all of these are a result of a defect in the folding of a specific protein (Kopito and Ron, 2000).

The most common feature to all conformational diseases is that the mutant proteins have a tendency to aggregate. Not only is the loss of function of the mutant protein important, but also it seems that the deposited aggregates are highly linked to and are responsible for the pathogenic effect. The ER protein folding machinery normally functions to prevent the misfolding of proteins and there are systems in place which are poised to respond to situations where increased protein misfolding occurs. These systems, which were already discussed include the abundance of ER chaperones, ER associated degradation, and the unfolded protein response. The abundant chaperones present in the ER are primarily responsible for the folding and assembly of proteins, however this system would obviously fail for a specific protein in which a mutation inherently perturbed the stability of the protein structure. The ERAD and UPR systems may only be suited for the intrinsic level of misfolded proteins that occur under normal cellular conditions or physiological stress situations and may not be able to cope with the load of misfolded protein brought about by genetic mutation. Thus the ER quality control pathway is very closely linked to conformational diseases. Many of these disease related proteins have been studied in relation to chaperone interactions, and specifically both wildtype and mutant forms of the CFTR protein have been shown to interact with calnexin (Chevet et al., 1999). An in-depth understanding of the ER molecular chaperones could provide insight into the role they play in genetic diseases and therefore may aid in potential treatment.

Calreticulin and the calreticulin deficient mouse

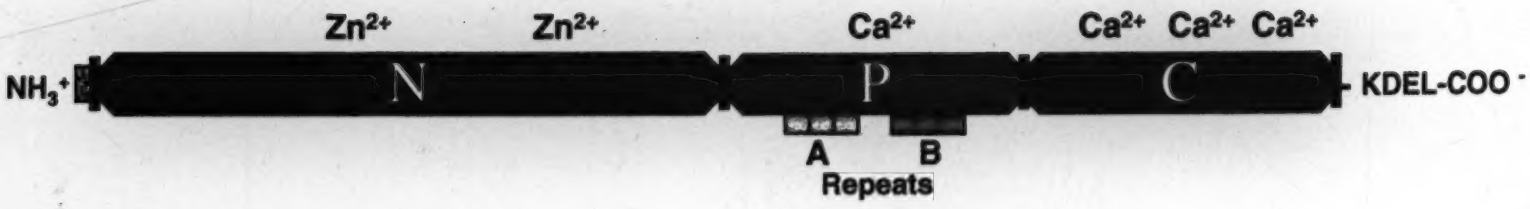
Although calreticulin is important as a molecular chaperone in the ER {Nauseef, 1995 #99}, it is also involved in many other cellular processes and many functions have been proposed for this protein (Ellgaard and Helenius, 2001; Michalak et al., 1999). Calreticulin was first isolated as a Ca^{2+} binding protein of the sarcoplasmic reticulum and was later identified as a major Ca^{2+} binding protein of the ER lumen (Johnson et al., 2001; Michalak et al., 1999; Milner et al., 1991; Ostwald and MacLennan, 1974). The human calreticulin protein consists of a 400 amino acid mature peptide. The protein is targeted to the ER by a 17-residue hydrophobic signal sequence that is eventually cleaved off (Michalak et al., 1999). The protein can be divided into three domains: the N-domain, which consists of 180 N-terminal residues; the P-domain, which consists of residues 181-290 and is rich in proline residues and contains two sets of repeated amino acid sequences; and the C-domain, which is the C-terminal portion of the protein and is rich in acidic amino acids (see Fig. 1-3 for summary of calreticulin). The P-domain of the protein binds to Ca^{2+} ions with a low capacity but high affinity (1 mol Ca^{2+}/mol protein, K_d = 1 μM), and the C-domain binds Ca^{2+} with a low affinity but high capacity (20-30 mol Ca^{2+}/mol protein, K_d = 2 mM) (Baksh and Michalak, 1991). These Ca^{2+} binding properties of calreticulin have resulted in its being believed to play a major role in intracellular Ca^{2+} homeostasis.

The number of functions in which calreticulin has been identified to play a role is vast. Due to its large capacity to bind Ca^{2+}, it is not surprising that calreticulin is involved in the regulation of Ca^{2+} homeostasis in the cell (Nakamura et al., 2001). Related to Ca^{2+} homeostasis is the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) which is a Ca^{2+} transporter found in the ER membrane. Calreticulin has been shown to affect the activity of SERCA by regulating its Ca^{2+} transport activity (Camacho and Lechleiter, 1995; John et al., 1998). Ca^{2+} release from the ER occurs through the Inositol-trisphosphate/ Ryanodine receptors (IP_3/RyR) by agonist mediated stimulation. Calreticulin has also been reported to affect Ca^{2+} release via this pathway and thus may regulate the activity of these receptors (Mesaeli et al., 1999). In addition to its ER Ca^{2+} related functions, calreticulin has also been reported to be involved in many functions outside of the ER. Calreticulin has been reported to modulate cell adhesion (Rojiani et al., 1991), integrin-dependent Ca^{2+} signalling (Coppolino et al., 1997), steroid-sensitive gene expression (Burns et al., 1994; Michalak et al., 1996), as well as cellular sensitivity to apoptosis (Nakamura et al., 2000). These are only a few of the functions calreticulin has been shown to be involved in, and evidence exists for its role in many other cellular functions such as nuclear export (Holaska et al., 2001) and in various aspects of the immune system (Johnson et al., 2001). The major controversy concerning calreticulin in being involved in all these functions however, is the mechanisms by which calreticulin can participate in functions outside the ER,

since calreticulin has only been found to be localized to the lumen of the ER and there is no solid evidence for it being found elsewhere (Michalak et al., 1999).

Figure1-3. The structure and function of calreticulin. Calreticulin is a 46 kDa Ca^{2+} binding chaperone found in the ER lumen. The three structural and functional domains of calreticulin are N, P, and C. The extreme N-terminus contains a hydrophobic ER-targeting signal sequence. The N-domain is highly conserved and contains Zn^{2+} binding sites. The P domain is the proline rich region and contains the high affinity Ca^{2+} binding site. The C domain is rich in acidic amino acids and binds Ca^{2+} with low affinity but high capacity. The N and P domains are responsible for protein-protein and glycopeptide interactions respectively.

CALRETICULIN



Features:

- -ER lumenal protein
- -highly conserved among species
- -contains signal sequence for ER targeting
- -high affinity/capacity Ca^{2+} binding protein
- -contains KDEL ER retention signal
- -consists of three domains: N, P, C

Functions:

- -regulation of Ca^{2+} homeostasis in ER
- -steroid sensitive gene expression
- -involved in apoptosis
- -cell adhesion
- -lectin-like protein chaperone

Figure 1-3

Since calreticulin is involved in a variety of important cellular functions, it was anticipated that the calreticulin deficient mouse would not be viable. Indeed, the calreticulin knockout mouse, created by the homologous gene recombination technique, is embryonically lethal at day 14.5 (Mesaeli et al., 1999). The major phenotypic abnormality of the calreticulin knockout mouse is the failure of absorption of the umbilical hernia, however histological analysis showed major lesions in the heart with a marked decrease in ventricular wall thickness, suggesting that the mouse most likely dies of a defect in development of the heart (Mesaeli et al., 1999). The calreticulin gene is activated during cardiac development and the amount of protein decreases in the newborn heart (Mesaeli et al., 1999), and is scarcely present in the adult heart (Tharin et al., 1996), indicating a probable important role for calreticulin during cardiac development. GRP94, another major ER lumenal Ca^{2+} binding chaperone is also up regulated during early cardiomyogenesis (Barnes and Smoak, 1997), suggesting that ER chaperones in general play an important role in the formation of the heart. The chaperone activity of calreticulin and in particular the calreticulin/calnexin cycle may be essential *in vivo* since the calreticulin knockout mouse did not survive to birth (Mesaeli et al., 1999).

Thesis Objectives

In this study, mouse embryonic fibroblasts (MEF) isolated from calreticulin deficient mice embryos were used to study the effect of calreticulin deficiency on the ER quality control system. The study of the ER chaperone system was accomplished by analyzing the changes in cellular protein levels of various ER protein folding enzymes in calreticulin deficient cells, and studying the effect of calreticulin deficiency on the function of several important ER chaperones.

The specific research objectives were:

- To analyze and identify the changes in the expression of various ER chaperones/protein folding enzymes in calreticulin deficient cells

- To determine if the change in the ER system due to the loss of calreticulin resulted in the unfolded protein response

- To analyze the effect of calreticulin deficiency on the chaperone function of calnexin, the functional homologue of calreticulin, as well several other important ER chaperones

- To attempt to identify alternative pathways for ER protein folding and quality control in the absence of calreticulin

The proposed work should provide important, new information into the function of ER chaperones. This should lead to a greater understanding and an elucidation of the protein folding pathway of secretory proteins in the ER. Such findings should provide us with greater insight into how chaperones function and may lead to a greater understanding of the role of chaperones in human protein conformational diseases.

Table 1-1. Protein folding factors of the endoplasmic reticulum

Name of protein	Type	Family	MW	function/mechanism	Reference
BiP	soluble	HSP70	78 kDa	peptide binding to hydrophobic sequences	Haas,I.G.,and Wabl,M. 1983
GRP94	soluble	HSP90	94 kDa	peptide binding	Koch., G. *et al*, 1986
PDI	soluble	thiol oxidoreductase	60 kDa	disulfide bond formation	Freedman, R.B. 1989
ERp57	soluble	thiol oxidoreductase	57 kDa	disulfide bond formation	Lewis, M.J. *et al*, 1986
ERp72	soluble	thiol oxidoreductase	72 kDa	disulfide bond formation	Lewis, M.J. *et al*, 1985
Calreticulin	soluble	lectin chaperone	46 kDa	lectin binding	Nauseef, W.M. *et al*, 1995
Calnexin	membrane bound	lectin chaperone	65 kDa	lectin binding	Ware, F.E. *et al*, 1995
Glucosidase I	membrane bound	glycan modifying enzyme	92 kDa	terminal glucose trimming	Kalz-Fuller, B. *et al*, 1995
Glucosidase II	soluble	glycan modifying enzyme	162 kDa	terminal glucose trimming	Ray, M.K. *et al*, 1991
UGGT	soluble	glycan modifying enzyme	170 kDa	glucosyl transferase	Parker, K. *et al*, 1995

MATERIALS AND METHODS

Reagents and Materials

Dulbecco's Modified Eagles Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and 0.05% Trypsin-EDTA solution were obtained from Invitrogen Life Technologies. Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and molecular weight markers were from Bio-Rad. The proteins used in these markers were myosin (200,000 Da), phosporylase b (97,400 Da), bovine serum albumin (66,000 Da), ovalbumin (46,000 Da), carbonic anhydrase (30,000 Da), and lysozyme (14,300 Da). For SDS-PAGE gels involving radiolabelled samples (see Cell Labelling and Immunoprecipitation), [^{14}C]-methylated protein standards (10-100 μCi/mg) of the same composition as above were used and obtained from Amersham Pharmacia Biotech. Triton X-100 detergent was form Bio-Rad. Protein A-sepharose CL-4B beads and Redivue [^{35}S]-Promix (containing L-[^{35}S]methionine and L-[^{35}S]cysteine; 14.3mCi/mL) were from Amersham Pharmacia Biotech. CHAPS (3-[(3-cholamidopropyl)dimethylammonio]1-propanesulfonate), tunicamycin, castanospermine, ALLN (N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal), L-cysteine, and L-methionine were all from Sigma. All other chemicals were from Sigma unless otherwise noted. All chemicals were of the highest grade available.

Antibodies

Rabbit anti-calnexin was a gift from Dr. J. Bergeron (McGill University, Montreal, Quebec). Rabbit anti-calreticulin was from Dr. W. Nauseef (University of Iowa, Iowa City, Iowa), and rabbit anti-BiP from Dr. L. Hendershot (Saint Jude Children's Research Hospital, Memphis, Tennessee). Rabbit anti-UGGT, rabbit anti-glucosidase IIα, and rabbit anti-glucosidase IIβ were from Dr. D. Thomas (McGill University, Montreal, Quebec). Rabbit anti-PDI was from Dr. S. Fuller (European Molecular Biology Laboratory, Heidelberg, Germany). Rabbit anti-PERK and mouse anti-IRE1 antibodies were from Dr. R.J. Kaufman (University of Michigan, Ann Arbor, Michigan). Rat anti-Grp94, and rabbit anti-KDEL raised against the QAKDEL synthetic peptide were previously made in Dr. M. Michalak's laboratory (University of Alberta, Edmonton, Alberta). Rabbit anti-ERp57, and rabbit anti-ERp72 were purchased from Stress Gene. Horseradish peroxidase-conjugated goat anti-rabbit IgG was from Jackson ImmunoResearch Laboratories.

Generation of Cell Lines and Cell Culture

Wildtype and calreticulin deficient mouse embryonic fibroblasts used in this study were derived from genotyped 10 day old mouse embryos (Mesaeli et al., 1999). Embryos were mechanically dissociated and tissue washed with PBS

(137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot H_2O$, 1.4 mM KH_2PO_4, pH 7.3). Cells were dissociated from tissue by incubating tissue in 0.05% Trypsin-EDTA solution at 37 °C for several minutes at a time and agitating in between. The suspension was then centrifuged at 4800 rpm for 1 min to remove debris and the supernatant containing cells was placed in 6-well tissue culture plates and incubated for 2 days at 37 °C. Cells were then passaged several times and grown to obtain more cells. Primary cell cultures were immortalized for experiments by stably transfecting with a plasmid containing the SV40 T antigen. To do this, cells were transfected with the pSV7 plasmid using the calcium phosphate method. DNA for transfection was purified using column chromatography (Qiagen Inc.) and 10 μg DNA was added per 10 cm plate to 50 % confluent cells. After 1 day, media serum concentration was reduced from 10% to 5% and transformants were selected on the basis of rapid colony formation after 2-3 weeks of growth in DMEM containing 5% FBS. Cells were passaged in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin, and maintained in this media under standard tissue culture conditions (5% CO_2 and humidity).

Cell Labeling and Immunoprecipitation

Wildtype and calreticulin deficient MEF's were grown to approximately 80% confluency in 100 mm plates. Cells were washed with PBS and incubated

with cysteine/methionine free DMEM for 45 min (starvation period). Cells were then pulsed with ^{35}S labelled cysteine/methionine to label newly synthesized protein by adding 137.5 μCi Redivue [^{35}S]-Promix in 1 mL of starvation media per dish and incubating under standard tissue culture conditions for 15 min. Radioactive media was then removed and cells were washed with PBS and chased for various times with DMEM supplemented with 5 mM each of unlabelled cysteine and methionine. After chase, cells were washed twice with ice cold PBS and once with cold HBS (50 mM Hepes, 200 mM NaCl, pH7.6).

For immunoprecipitation, 500 μL lysis buffer was added to each 10 cm plate containing pulse-labelled cells. Lysis buffers were prepared containing a protease inhibitor cocktail (0.5 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, and 5 μL "SL protease inhibitors"/10 ml buffer [0.5 μg N-[N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine/mL, 0.1 μg of aprotinin/mL, 0.5 μg leupeptin/mL, 0.5 μg pepstatin/mL, 50 μg L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride and L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone/mL, 0.1 μg (4-amidinophenyl)-methanesulfonyl fluoride/mL, 50 μg phosphoramidon/mL]). The lysis buffer used for immunoprecipitations with all antibodies except rabbit anti-calreticulin was 2% CHAPS in HBS. For the rabbit anti-calreticulin antibody the lysis buffer used was 1% Triton X-100 in TBS (50 mM Tris, 150 mM NaCl, pH 7.5). After addition of lysis buffer, cells were scraped with a rubber policeman and pipetted into microfuge tubes. Cell lysates were clarified by centrifugation in an Eppendorf 5415C microfuge and F-45-18-

11 rotor at 14,000 for 10 min and 4°C. Supernatants were transferred to new tubes and pre-cleared by adding 60 µL 10% Protein A-Sepharose suspension (in HBS) and rotating in a microfuge tube rotator at 10 rpm and 4°C for 30 min. Immunoprecipitation was conducted by adding the appropriate antibody and 100 µL 10% Protein A-sepharose together and rotating tubes as above for 4 hours to overnight. Beads were pelleted by centrifuging briefly for 10 sec at 14,000 rpm (Eppendorf 5415C microfuge and F-45-18-11 rotor) and then washed three times with 200 µL 1% Chaps/HBS and once with HBS. Beads were resuspended in 20 µL SDS-PAGE sample buffer, boiled for 3 min and centrifuged at 14,000 rpm (Eppendorf 5415C microfuge and F-45-18-11 rotor) for 1 min to pellet beads. The supernatant was loaded and run on an 8% SDS-PAGE gel. The gel was dried and labelled protein bands were visualized using phosphorimaging. Dried gels were placed under a FUJI Type BAS-IIIs imaging plate for an exposure time of 48 hours. The imaging plate was scanned using a FUJI BAS1000 Phosphorimager and images were processed using Image Gauge V3.0 software. For experiments involving various treatments, castanospermine (0.5 mM), tunicamycin (20 µg/mL), or N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN, 50 µg/mL) were included in the media used throughout the starvation, radioactive pulse, and chase steps.

Isolation of nuclear membrane from fibroblasts

Wildtype and calreticulin deficient cells grown to confluency in 10 cm tissue culture plates were washed three times with ice cold PBS. Five plates of each cell type were scraped into a total of 2 mL TKM buffer (50 mM Tris, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$) + 0.25 M sucrose. Cell suspension was mechanically homogenized and aliquoted into 6 swing-bucket ultracentrifuge tubes, then 2 mL TKM + 2.3 M sucrose was added to each tube and mixed by inversion. The mixture was underlined with 1 mL TKM + 2.3 M sucrose at the bottom of the tubes using a syringe. Tubes were then centrifuged in an SW41T1 swing-bucket rotor at 39,000 rpm 4°C, for 30 min in a Beckman L8-70M Ultracentrifuge. After centrifugation, the interface pellet was pipetted into 15 mL Corex tubes, TKM buffer was added to 11 mL to each sample, mixed, and centrifuged at 14,000 rpm, 4 °C, for 120 min using a JA-17 rotor in a Beckman J2-21M centrifuge. After centrifugation, supernatant was discarded and pellets were resuspended in 3 mL TKM buffer. 1 mL TKM + 2% Triton X-100 was added to each sample, and the mixture was centrifuged at 800x g, 4°C, for 5 min. The pellet, containing the inner nuclear membrane was resuspended in 500 µL TKM buffer. The supernatant, containing outer nuclear membrane, was precipitated with acetone by bringing the volume up to 20 mL with acetone and placing overnight at –80°C. Samples were then centrifuged at 10,000 rpm (JA-17 rotor, Beckman J2-21M centrifuge) 4°C, for 1 hour in a JA-17 rotor. Pellets of outer nuclear membrane were dried and resuspended in 250 µL TKM buffer and protein concentration was determined on all samples.

SDS-Polyacrylamide gel electrophoresis and Western blotting

Wildtype and calreticulin deficient cells grown to confluency on 10 cm dishes were lysed with 300 μL New RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing a protease inhibitor cocktail (as above). Cells were scraped into microfuge tubes and lysates clarified by centrifuging at 14,000 rpm (Eppendorf 5415C microfuge, F-45-18-11 rotor) for 5 min. Supernatants were transferred to new tubes and protein concentration determined on the cell extracts. Samples were then diluted to appropriate concentration in SDS-PAGE sample buffer and the indicated amounts of total protein were loaded onto gels. For all antibodies except mouse anti-IRE1α, total cell extract was used. For the mouse anti-IRE1α antibody, proteins present in the isolated outer nuclear membrane were separated by SDS-PAGE.

SDS-PAGE was performed according to Laemmli (Laemmli, 1970). 10% and 15% acrylamide gels were used. After gel electrophoresis, for detection of separated proteins the gels were electrophoretically transferred to nitrocellulose membrane using a semi-dry transfer apparatus (Towbin et al., 1979).

After transfer, nitrocellulose membranes were stained with Ponceau S (Sigma) to verify that the protein transfer had occurred. The membranes were blocked in 5% skim milk powder dissolved in PBS for 30 min at room temperature with shaking. The blocked membranes were then incubated with primary antibody diluted in 1% milk powder in PBS for 1 hour. Dilutions of individual antibodies used for Western blots are listed in Table 2-1. After

incubation with the primary antibody the membranes were washed twice for 10 min each in 0.05% tween-20 in PBS followed by one 10 minutes wash in PBS alone. The membranes were then incubated with the appropriate horseradish peroxidase conjugated secondary antibody diluted 1:10,000 in 1% milk powder in PBS for 1 hour. The wash steps were then repeated as above. Detection of the bound antibodies was by the chemiluminescent ECL detection system (Amersham Pharmacia Biotech). X-ray film (FUJI) was placed on membrane after ECL addition for the appropriate time and developed using a Kodak X-OMAT 200 Processor. For quantification, x-ray films were scanned and saved as pict files, then protein bands were quantified using Image Gauge V3.0 software. The quantitation program produced arbitrary numbers for each band, and from these values, ratios were calculated for each protein tested as the value for calreticulin deficient cell type divided by the value for the wildtype cell type. These ratios were calculated for each amount of protein on the gel and from several immunoblots done on various days, and averaged over all experiments. These averages were plotted on bar graphs with error bars showing the standard deviations. A t-Test was performed on all the data sets to indicate statistical significance at the 0.05 significance level, and those which passed this test are indicated in the graph.

Determination of protein concentration

Protein concentration was determined by the method Bradford (Bradford, 1976). Bovine serum albumin was used as a standard in these assays.

Table 2-1. Dilutions of antibodies used for Western blots.

Antibody	dilution in 1% milk/PBS solution
rabbit anti-KDEL	1:100
rabbit anti- calnexin	1:1000
rabbit anti-PDI	1:500
rabbit anti-BiP	1:500
rabbit anti-ERp57	1:1000
rabbit anti-ERp72	1:1000
rabbit anti-glucosidase IIa	1:100
rabbit anti-glucosidase IIb	1:100
rat anti-GRP94	1:1000
rabbit anti-UGGT	1:500
rabbit anti-PERK	1:2000
mouse anti-IRE1	1:1000

EXPRESSION OF ER FOLDING PROTEINS IN CALRETICULIN DEFICIENT CELLS

Introduction

A variety of quality control mechanisms exist in the ER to ensure that newly synthesized secretory proteins are folded properly and obtain their correct functional conformation. The most commonly observed ER quality control mechanism, and perhaps among the most important, is the association of newly synthesized proteins with ER chaperones and folding enzymes (Ellgaard et al., 1999). This function not only assists in the folding and assembly process, but also serves as a retention mechanism for immature proteins. In the ER, this primary level of quality control is exerted by a functionally redundant system within which individual ER chaperones bind their substrates differently.

BiP binds to exposed hydrophobic determinants on newly synthesized polypeptides (Flynn et al., 1991); GRP94 protein also functions by peptide binding (Nieland et al., 1996). The thiol oxidoreductase family of proteins, namely PDI, ERp57, and ERp72, catalyze the oxidation and isomerization of disulfide bonds (Huppa and Ploegh, 1998; Molinari and Helenius, 1999). Both calnexin and calreticulin bind to newly synthesized proteins and glycoproteins via specific N-linked oligosaccharide tags indicative of incomplete folding. In addition to the typical chaperones required for folding, calnexin and calreticulin cooperate with several independently acting enzymes that regulate substrate binding. These include glucosidases I and II, responsible for glucose trimming, and UGGT which catalyzes the readdition of glucose residues.

For primary quality control, there is a multitude of proteins involved and a variety of mechanisms by which each individual protein carries out its function. The overall process of chaperoning, however, is thought to be an inter-related and redundant system, in which many of the chaperones cooperate together. In the case of calnexin and calreticulin, they participate in a cycle of substrate binding, which involves the glycoprotein modifying enzymes glucosidases I and II, and UGGT (Helenius et al., 1997). Also the thiol oxidoreductases (PDI, ERp57) have been shown to interact with calnexin, calreticulin, and BiP in the folding of proteins (Mayer et al., 2000; Molinari and Helenius, 1999; Oliver et al., 1999; Zapun et al., 1998). The inter-relation of the ER chaperones was established by showing that calnexin exists in complexes with calreticulin, BiP, GRP94, and other proteins (Tatu and Helenius, 1997). The redundancy of the chaperone system is illustrated in a study where if one chaperone is prevented from interacting with an incompletely folded protein, then another most likely will take over (Zhang et al., 1997), although the extent of this is not known and is the subject of this study.

Given the close relationship and redundancy in the ER chaperone system, what is the specific function or importance of individual proteins in the system? Calreticulin being an important protein in that it has many known and proposed functions (Johnson et al., 2001; Michalak et al., 1999) has been proposed to play an important role in the chaperone system. Thus this might suggest that the removal of calreticulin from the ER system would have a great impact on the

function of other ER chaperones. In this section, the effect that the loss of calreticulin has on the expression of ER chaperones is studied. This would provide insight into the overall pathway for protein folding in the ER.

Results

To determine if the absence of calreticulin changed the expression of various ER chaperones, Western blot analyses were done on cell extracts of wildtype and calreticulin deficient fibroblasts with antibodies to the various ER proteins. To obtain a reliable estimate of the quantitation of the protein bands, varying amounts of total cellular protein were loaded onto the polyacrylamide gels as indicated in the figures.

Figures 3-1 and 3-2 show the effect of calreticulin deficiency on the expression of proteins of the calnexin/calreticulin cycle. In Figure 3-1 the calreticulin protein band is present in the wildtype cell extract, however no calreticulin protein band is observed in the extract from calreticulin deficient cells. This confirms that calreticulin is absent in embryonic fibroblasts isolated from calreticulin knockout mice as previously determined (Mesaeli et al., 1999; Nakamura et al., 2000). Figure 3-2 shows the expression patterns of calnexin, the α and ß-subunits of glucosidase II, and UGGT in wildtype and calreticulin deficient cells. Quantification analysis of the level of protein showed that in the

calreticulin knockout cells, expression of calnexin was increased by an average of 30%, (+/-4%) and the α and ß-subunits of glucosidase II were increased by 10% (+/-3%) and 40% (+/-8%), respectively. Since the change in levels of the two subunits of glucosidase II are not the same, the overall change in this protein cannot be accurately determined. Nevertheless, since both subunits showed increased levels in calreticulin deficient cells, this suggests that glucosidase II is upregulated in these cells. UGGT was difficult to detect with the antibody and required large amounts of cell lysate to visualize the band. Thus for UGGT only, 50 μg of total protein was loaded onto the gel. Quantification of these immunoblots indicated that the level of UGGT was unchanged in calreticulin deficient cells (Fig. 3-2). The increased levels of calnexin, and glucosidase II subunits suggest that the response to the absence of calreticulin is an upregulation of the various chaperones/modifying enzymes involved in the calnexin/calreticulin chaperone cycle.

The change in expression of other types of chaperones was also examined to see if these were affected by the loss of calreticulin. Figure 3-3 shows the expression of BiP and GRP94, ER chaperones not involved in folding of glycosylated proteins. Both of these proteins were significantly upregulated in calreticulin deficient cells. Quantitation of the protein bands showed that both BiP and GRP94 were increased by 40% (+/-7 and 6% respectively) in these cells (Fig. 3-3). Shown in Figure 3-4 are the Western blots of the thiol oxidoreductase family of chaperones. Quantitation of the protein bands revealed that expression

of PDI was decreased by 20% (+/-3%) in calreticulin deficient cells compared to the wildtype. ERp57 and ERp72, however, were both upregulated in calreticulin deficient cells by 20% (+/-5%) and 5% (+/-1%) respectively. (Fig. 3-3). The significant decrease of PDI in the calreticulin deficient cells may be an interesting observation, although it is difficult to explain since the other members of the thiol oxidoreductase family of proteins are clearly upregulated and the function of these proteins are similar to those of PDI.

The general trend of increased protein chaperones in the calreticulin knockout cells suggested that these cells might be under stress. An upregulation of protein chaperones of the ER, and specifically an increase in BiP protein, is an indicator of cellular stress and is a result of the unfolded protein response pathway (UPR). The UPR pathway is mediated by the transmembrane kinase signalling factors Ire1 and PERK (Harding et al., 1999; Tirasophon et al., 1998). These kinases are responsible for transmitting the stress signal from the ER to the nucleus. Since the general upregulation of protein chaperones in the calreticulin knockout cells indicated an activation of the UPR pathway, we investigated the effect of calreticulin-deficiency on the expression and function of both Ire1 and PERK. Initial attempts at Western blot analysis with anti-Ire1α antibodies failed for cell extracts of both wildtype and calreticulin deficient cells. PERK, however, was readily detectable in total cell extracts. Ire1α was detected by Western blot in purified inner nuclear membrane fractions. Quantitation of the protein bands observed showed that in the calreticulin deficient cells Ire1α was increased by

200% in these membranes than the wild type fibroblasts (Fig. 3-5). Western blot analysis with anti-PERK antibodies and quantitation of the protein bands revealed that PERK was increased by over 500% in calreticulin deficient cells as compared to the wildtype (Fig. 3-5). These results suggest an active UPR pathway in the calreticulin deficient cells.

A summary of the results of the of the quantification data for the ER chaperone protein expression levels in wild type and calreticulin deficient cells is represented in Figure 3-6 and Table 3-1.

Conclusions and Discussion

In this part of the study we showed that the deletion of the ER chaperone calreticulin had significant changes in the expression profile of several other ER proteins. Both calnexin and glucosidase II showed increased levels in calreticulin deficient cells. This might suggest that the loss of calreticulin resulted in the impairment of the calnexin/calreticulin cycle and that the upregulation of these proteins was a mechanism of compensating for the loss of calreticulin. The problem arises where the lectin chaperoning cycle may not actually be able to function correctly without calreticulin, thus increases in the proteins of the cycle may be futile. Interestingly, we also determined in this study that significant increases occur in levels of chaperones not directly involved in the

calreticulin/calnexin cycle, namely BiP and GRP94. Both BiP and GRP94 proteins showed noticeable increases in the calreticulin knock out cells as compared to the wild type. This result may imply that these proteins may be responsible for compensating for the loss of calreticulin. An increase in BiP in the calreticulin deficient cells also indicates that these cells may be under stress, as an upregulation of BiP is an indicator of the unfolded protein response (UPR) (Gething, 1999). Indeed the results in this study showed that the UPR was activated in the calreticulin deficient cells as shown by large increases in the amounts of Ire1α and PERK, proteins which induce the signal of the UPR pathway. Since BiP synthesis increases during conditions that lead to the accumulation of unfolded polypeptides in the ER (Gething, 1999), this suggests that the accumulation of unfolded proteins may be the result of the loss of calreticulin. This would indicate that calreticulin plays a vital role as a chaperone and that the degree of redundancy among chaperones is lower than previously thought since there are deficiencies in the folding pathway when one chaperone is absent.

Figure 3-1. Expression of calreticulin in wildtype and calreticulin knockout cells. Whole cell extract was used from wildtype and calreticulin deficient cells for Western blot analysis. The indicated amounts of total protein of cell lysate were loaded onto SDS-polyacrylamide gels consisting of 10% acrylamide, and immunoblotted with rabbit anti-calreticulin antibody. Horseradish peroxidase conjugated secondary antibody and enhanced chemiluminescence were used to detect immunoreactive protein bands by exposure to X-ray film.

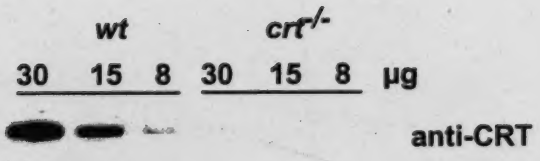
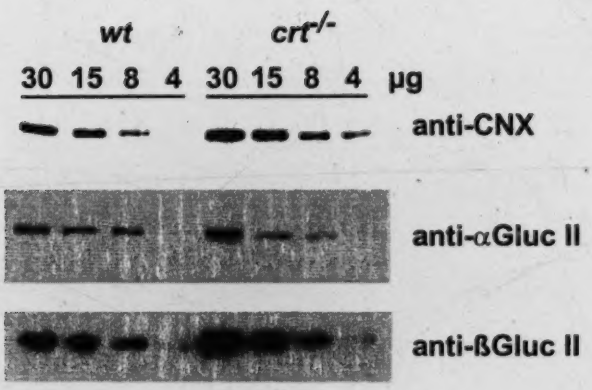


Figure 3-1

Figure 3-2. Expression of protein of the calreticulin/calnexin cycle in wildtype and calreticulin knockout cells. Whole cell extract was used from wildtype and calreticulin deficient cells for Western blot analysis. The indicated amounts of total protein of cell lysate were loaded onto SDS-polyacrylamide gels consisting of 10% acrylamide, and immunoblotted with rabbit anti-calnexin, rabbit anti-glucosidase IIα, rabbit anti-glucosidase IIβ, and rabbit anti-UGGT antibodies. For the UGGT immunoblot, 50 μg total protein was loaded onto SDS-polyacrylamide gels consisting of 7.5% acrylamide. Horseradish peroxidase conjugated secondary antibody and enhanced chemiluminescence were used to detect immunoreactive protein bands by exposure to X-ray film. To quantify the amount of protein, the protein bands from each lane were used as well as from additional immunoblots not shown, as described in Materials and Methods. For CNX protein a total of 5 separate immunoblots were used for quantification, for glucosidase IIβ 6 blots were used, and for glucosidase IIα and UGGT a total of 4 blots were used. Shown are the best representative immunoblots. CNX, calnexin; UGGT, UDP-glucose:glycoprotein glucosyltransferase.


wt
crt-/-
30 15 8 4 30 15 8 4 µg
anti-CNX
anti-αGluc II
anti-ßGluc II

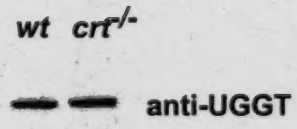

wt crt-/-
anti-UGGT

Figure 3-2

Figure 3-3. Expression of BiP and GRP94 in wildtype and calreticulin knockout cells. Whole cell extract was used from wildtype and calreticulin deficient cells for Western blot analysis. The indicated amounts of total protein of cell lysate were loaded onto SDS-polyacrylamide gels consisting of 10% acrylamide, and immunoblotted with rabbit anti-BiP and rat anti-GRP94 antibodies. Horseradish peroxidase conjugated secondary antibody and enhanced chemiluminescence were used to detect immunoreactive protein bands by exposure to X-ray film. To quantify the amount of protein, the protein bands from each lane were used as well as from additional immunoblots not shown, as described in Materials and Methods. For BiP, five separate immunoblots were used for quantification, and for GRP94, a total of 7 blots were used. Shown are the best representative immunoblots.

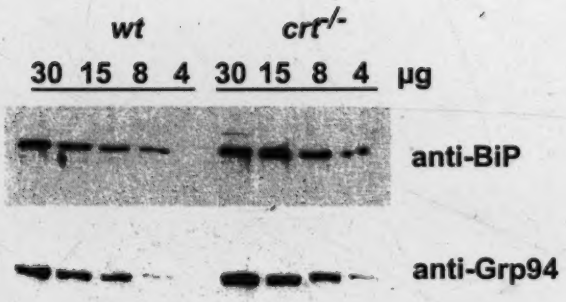


Figure 3-3

Figure 3-4. Expression of the thiol oxidoreductase family of proteins in wildtype and calreticulin knockout cells. Whole cell extract was used from wildtype and calreticulin deficient cells for Western blot analysis. The indicated amounts of total protein of cell lysate were loaded onto SDS-polyacrylamide gels consisting of 10% acrylamide and immunoblotted with rabbit anti-PDI, rabbit anti-ERp57, and rabbit anti-ERp72 antibodies. Horseradish peroxidase conjugated secondary antibody and enhanced chemiluminescence were used to detect immunoreactive protein bands by exposure to X-ray film. To quantify the amount of protein, the protein bands from each lane were used as well as from additional immunoblots not shown, as described in Materials and Methods. For PDI, six separate immunoblots were used for quantification, for ERp57 4 blots were used, and for ERp72 a total of 6 immunoblots were used. Shown are the best representative immunoblots. PDI, protein disulfide isomerase.

Figure 3-4

Figure 3-5. Expression of Ire1α and PERK in the unfolded protein response in wildtype and calreticulin knockout cells. For PERK analysis whole cell extract was used from wildtype and calreticulin deficient cells for Western blot. Fifty μg of total protein of cell lysate was loaded onto SDS-polyacrylamide gels consisting of 10% acrylamide, and immunoblotted with rabbit anti-PERK antibody. For Ire1α, inner nuclear membranes were isolated from wildtype and calreticulin deficient cells, and 50 μg of total protein was loaded and separated by SDS-PAGE consisting of 10% acrylamide, and immunoblotted with mouse anti-Ire1α antibody. Horseradish peroxidase conjugated secondary antibody and enhanced chemiluminescence were used to detect immunoreactive protein bands by exposure to X-ray film.

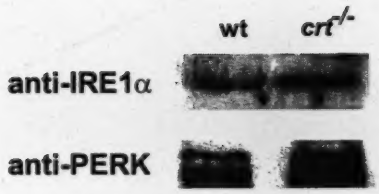


Figure 3-5

Figure 3-6. Average CRT deficient to WT ratio of relative protein expression levels as determined by Western blot. Western blots were conducted for each protein using the appropriate antibody and protein bands were quantified using Image Gauge V3.0 software as described. The ratios were calculated as the value for calreticulin deficient cell type divided by the value for the wildtype cell type. The bar graph shows the average results of several experiments. The wildtype protein levels would be represented by the value of 1. For Gluc-IIβ and ERp72 proteins, the average from 6 separate Western blots were used; for Gluc-IIα, UGGT, and ERp57 4 blots were used; for CNX and BiP 5 blots were used; for GRP94 and PDI 7 blots were used. The error bars represent the standard deviations of the averages. The bars marked with an asterisk (*) indicate that these values are statistically significant according to the t-Test at the 0.05 significance level.

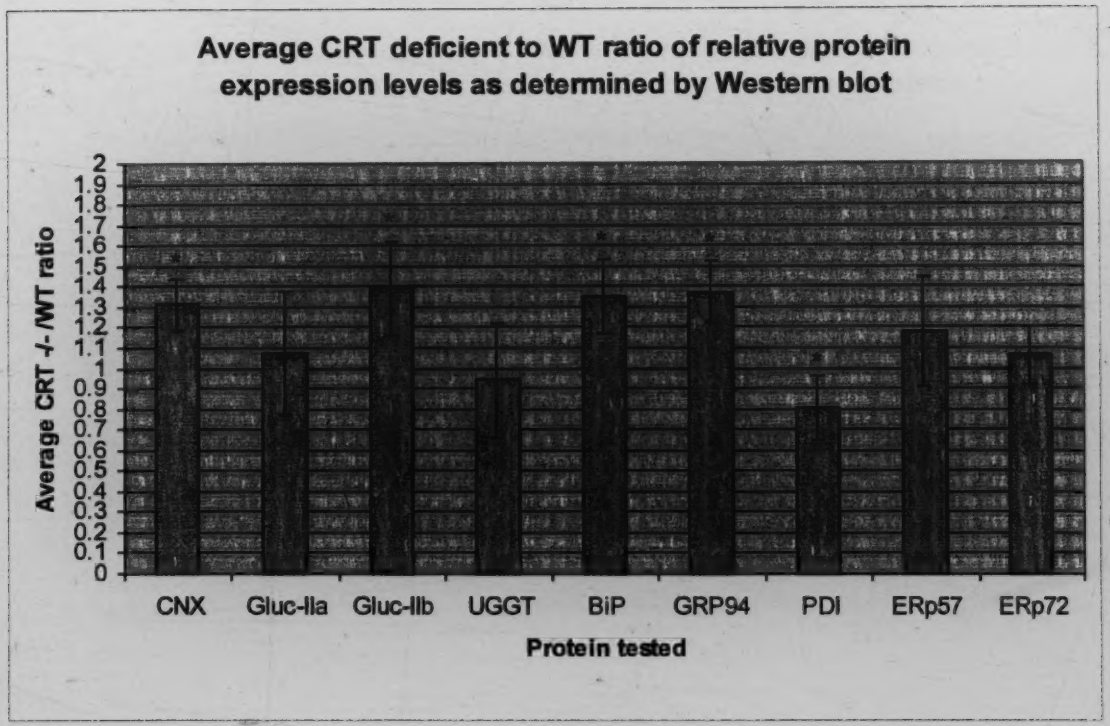

Average CRT deficient to WT ratio of relative protein expression levels as determined by Western blot
Average CRT -/- /WT ratio
2
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
CNX
Gluc-IIa
Gluc-IIb
UGGT
BiP
GRP94
PDI
ERp57
ERp72
Protein tested

Figure 3-6

Table 3-1. Summary of protein expression levels in calreticulin deficient cells as determined by Western blot analysis.

Protein examined	general ER function	change observed relative to wildtype
calreticulin	CNX/CRT cycle	N/A
calnexin	CNX/CRT cycle	increased 30%
glucosidase IIα	CNX/CRT cycle	increased 10%
glucosidase IIβ	CNX/CRT cycle	increased 40%
UGGT	CNX/CRT cycle	no change
BiP	general ER chaperone	increased 40%
GRP94	general ER chaperone	increased 40%
PDI	thiol oxidoreductase	decreased 20%
ERp57	thiol oxidoreductase	increased 20%
ERp72	thiol oxidoreductase	increased 5%
Ire1	unfolded protein response	increased 200%
PERK	unfolded protein response	increased 500%

A STUDY OF ER CHAPERONE FUNCTION IN CALRETICULIN DEFICIENT CELLS

Introduction

In the previous part of this study, the effect of calreticulin deficiency on the expression of ER chaperones and chaperone-associated proteins was studied. It was observed that several changes in ER protein expression occurred when calreticulin was not present. The majority of these changes were an increase in expression of chaperones, specifically those of the calreticulin/calnexin cycle and of BiP and GRP94 which are not involved in this cycle. From these results we postulated that an increase in the amounts of these various proteins might be a compensatory effect to the loss of calreticulin. Thus it was necessary to study the effect of calreticulin deficiency on the function of ER chaperones. We therefore next investigated the effect of calreticulin deficiency on protein folding in the ER.

Since the chaperoning of proteins during folding requires direct interaction between the chaperone and the substrate (Helenius et al., 1997), and both calnexin and calreticulin have been shown to directly interact with many of their folding substrates (Ihara et al., 1999; Saito et al., 1999), an effective method to study the function of chaperones is to use immunoprecipitation and observe the set of co-precipitating proteins. These represent the substrates of the chaperone and can be analyzed in several ways such as identity and kinetics of association with the chaperone.

To analyze the interaction of newly synthesized cellular proteins with chaperone proteins in cells, the cells were pulse-labelled with [^{35}S]-methionine/cysteine mixture to radioactively label the newly synthesized protein. The cells were lysed using a non-denaturing detergent to ensure that protein complexes remained intact. The cell lysates were then subject to immunoprecipitation with antibodies to the various chaperone proteins to observe the co-precipitating substrates.

Results

Immunoprecipitation with anti-calnexin antibodies showed that, in agreement with previous studies, a variety of substrates ranging from 30- to 220-kDa transiently associated with calnexin (Fig. 4-1). The majority of these substrates were released within the first 60 min of chase (Fig. 4-1, wildtype), which correlates with previous pulse-chase studies that have shown that most substrates bind to calnexin for not more than 60 min (Hammond et al., 1994; Ou et al., 1993). In calreticulin deficient cells, the substrates that co-precipitated with calnexin were almost identical to those in the wild type cells. In these cells, however, the kinetics of substrate binding displayed striking differences (Fig. 4-1, *crt*$^{-/-}$). In the calreticulin knockout cells, the majority of the calnexin-associated substrates exhibited an enhanced turnover. Although this can be seen by examining the gels, the bands which are clearly visible that exhibit faster

dissociation in the calreticulin deficient cells are marked (Fig. 4-1, indicated by •). To quantitate the differences observed between the wildtype and calreticulin deficient cells, another gel in which the bands were less congested was used (Fig. 4-2). Protein bands from five positions were picked (180-, 60-, 47-, 34-, 27-kDa) and the amount of each was quantitated at each time point using Image Gauge software. The values were plotted in arbitrary units. Shown in Figure 4-2 are the plots of the quantitated protein bands. From this analysis it is apparent that in calreticulin deficient cells, substrate dissociation is much faster than in the wild type cells, with an average $t_{1/2}$ of association in the wildtype of 20 min and an average $t_{1/2}$ of 10 min in the calreticulin deficient cells.

To confirm that this increased substrate dissociation from calnexin was specifically due to the loss of calreticulin, calreticulin deficient cells were stably transfected with recombinant calreticulin, and substrate association with calnexin was examined in these cells. The back-transfected cells were pulse-labelled and followed by immunoprecipitation with anti-calnexin antibodies. A similar pattern of calnexin substrate binding and kinetics was observed in the back- transfected cells compared to the wild type cells, with the half-times of association of the major substrates restored to that of the wildtype (Fig. 4-1, wildtype; and Fig. 4-1 *crt*$^{-/-}$+rCRT). Thus by transfecting calreticulin back into the knockout cells, the wildtype phenotype can be restored. This indicates that the behaviour of calnexin substrates in the calreticulin knockout cells was specifically due to calreticulin deficiency (Fig. 4-1, compare wild type to *crt*$^{-/-}$+rCRT). Theseresults indicate that

calnexin chaperone function is impaired in the absence of calreticulin as shown by the accelerated release of calnexin substrates in calreticulin deficient cells. Evidently, calnexin cannot compensate for the chaperone requirements associated with calreticulin and this may, at least in part, explain why calreticulin deficiency is embryonic lethal (Mesaeli et al., 1999).

The binding of substrates to both calreticulin and calnexin is dependent upon the presence of monoglucosylated glycans on the substrate polypeptides (Helenius et al., 1997). These glycan moieties are added onto the growing nascent chain and then trimmed to the monoglucosylated form by glucosidases I and II. Glucosidases I and II are sensitive to inhibitory drugs such as castanospermine (CST). Thus by inhibiting the glucosidases, castanospermine prevents the binding of newly synthesized glycoproteins to the lectin-specific chaperones. This allows CST to be used as a tool to study the maturation of glycoproteins in the absence of binding to calnexin and calreticulin (Helenius et al., 1997). Conversely by preventing glycoprotein binding to calnexin and calreticulin, CST can also be used to study the function of these chaperones. Also the addition of glycan moieties to polypeptides at the specific sequence is subject to inhibition by the drug tunicamycin (TUN). Both castanospermine and tunicamycin are drugs that can be used to study the function of calnexin and calreticulin in the absence of glycoprotein substrate binding.

To examine what features of calnexin chaperone function were affected by the loss of calreticulin, we tested the effect of inhibitors of glycosylation and glucose trimming on calnexin substrate association in both wildtype and calreticulin deficient cells. First we used tunicamycin, an inhibitor of N-glycosylation. The cells were treated with the drug, and pulse-chased and immunoprecipitated with calnexin as with the previous experiments. The results obtained with tunicamycin treatment are shown in Figure 4-3. Treatment of the cells with tunicamycin caused a dramatic reduction of substrate binding to calnexin (compare Fig 4-1 wildtype, and Fig 4-3) and very few bands can be observed co-precipitating with calnexin under these conditions as is expected since most of calnexin's substrates are glycoproteins. Some protein bands, however, can be seen coprecipitating with calnexin under these conditions; these interactions may either be non-specific or may occur through non-glycan interactions. It is possible some of these bands represent specific calnexin substrates interacting through protein-protein interactions, as suggested by the transient nature of association (Fig. 4-3, open arrowhead). Calnexin has previously been reported to interact directly with polypeptides (Ihara et al., 1999). In the calreticulin deficient cells, this carbohydrate-independent substrate exhibited enhanced turnover and dissociation kinetics from calnexin (Fig. 4-3, open arrowhead) similar to the pattern of glycosylated substrates seen in untreated cells. Although this observation might suggest that calreticulin is necessary for both lectin and non-lectin functions of calnexin, data presented here is not conclusive of this because of the possibility that the substrates observed co-

precipitating with calnexin under tunicamycin treatment may be due to non-specific association. An appropriate standard control to reveal the level of non-specific associations for the calnexin immunoprecipitation experiments would be an immunoprecipitation from the same cells with a pre-immmune serum. However, this is not present in this study.

Treatment of the cells with castanospermine showed a similar pattern of pattern of coprecipitating proteins as that with tunicamycin (Fig. 4-4). The vast majority of substrates vanished when the cells were treated with castanospermine (compare Fig. 4-1 wildtype, and Fig. 4-4), confirming that calnexin mainly interacts with glycoproteins of the monoglucosylated form. That the pattern is almost identical to tunicamycin and that no additional bands are present indicates that calnexin does not bind substrates that are glycosylated but not of the monoglucosylated from. As with the tunicamycin treatment, under treatment with castanospermine calnexin picked up the non-glycoprotein substrate (Fig. 4-4, open arrowhead). Thus substrate also exhibited impaired binding kinetics in the calreticulin deficient cells. In summary, Figures 4-3 and 4-4 show that vast majority of substrates interacted with calnexin via the sugar moiety. Importantly, regardless of the nature of association with calnexin these proteins exhibited enhanced turnover and significantly shorter association time with calnexin in the calreticulin deficient cells.

The above experiments show that newly synthesized proteins dissociate faster from calnexin in the absence of calreticulin. Determining the fate of these

substrates would indicate how calreticulin might function in the chaperoning environment. To determine if the faster dissociation of substrates was due to increased degradation through the ER associated degradation pathway (ERAD), we studied the fate of the substrate proteins by treating wild type and calreticulin deficient cells with the proteasome inhibitor, N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN). After this treatment the cells were pulse-labelled and immunoprecipitated with anti-calnexin antibody. The results obtained with ALLN treatment are shown in Figure 4-5. Immunoprecipitation with anti-calnexin antibodies revealed that under these conditions there was an increased association of proteins with calnexin in calreticulin deficient cells, especially at the later chase times (Fig. 4-5, arrowheads). Since calnexin is responsible for binding and chaperoning unfolded proteins, this indicates that there was an accumulation of proteins that remained unfolded in the absence of calreticulin. The accumulation of unfolded substrates in the calreticulin knockout cells showed that there is a requirement for a functional calreticulin/calnexin pathway for the proper chaperoning and release of newly synthesized proteins. Substrates unaffected by ALLN treatment are either calnexin-specific and do not require calreticulin, or alternatively, for these particular substrates other ER chaperones may be able to compensate for the loss of calreticulin (see below). This experiment shows that without calreticulin some proteins cannot fold efficiently and thus are targeted for degradation, indicating that calreticulin plays an important role in the folding of proteins.

The dramatic change in the function of calnexin in the absence of calreticulin represents a deficiency in the folding of the substrate proteins since the majority of these proteins dissociate from calnexin at a faster rate and remain unfolded. The fact that calreticulin deficiency had an effect on how calnexin operates might suggest that these two chaperones share their folding substrates. In order to determine if many of the substrate proteins of calreticulin and calnexin were shared, fibroblasts were pulse-chase labelled and calreticulin was immunoprecipitated from these cells. The results of the calreticulin immunoprecipitation are shown in Figure 4-6. Figure 4-6 shows that there were numerous proteins associated with calreticulin in the wildtype cells ranging from 35- to 350-kDa. The majority of the low molecular weight bands (<66,000 Da), however, appear to be non-specific as these are also present in the calreticulin deficient cells. In agreement with earlier reports (Peterson et al., 1995) the vast majority of calreticulin specific substrates were proteins of a relatively high molecular weight and most of them were released from calreticulin 45 min post-chase (Fig. 4-6, wild type). When the calreticulin immunoprecipitation was done in the calreticulin knockout cells, the bands that were observed were non-specific as expected since there is no calreticulin present in these knockout cells (see Fig. 3-1). Comparison of the wildtype samples in the Figures 4-1 and 4-6 indicated that in mouse embryonic fibroblasts there were few substrates common to both calnexin and calreticulin and most of these were of high molecular weight (compare Figs. 4-1 wildtype and 4-6). This suggested that the majority of the substrate proteins might be unique to each chaperone, and suggested that the

impairment of calnexin's function in the absence of calreticulin may not be due loss of substrate interaction with calreticulin, but that calreticulin may act in a different manner to influence the function of calnexin.

We next wanted to study the effect of calreticulin deficiency on the function of other ER lumenal chaperones. Since BiP defines a separate ER chaperone system from the calreticulin/calnexin pathway, we tested if the absence of calreticulin and impaired function of calnexin might have any affect on the activity of BiP as a chaperone. Wildtype and calreticulin deficient cells were pulse-chase labelled as before and the cell lysates were used to immunoprecipitate BiP. The results of this procedure are shown in Figure 4-7; we observed that BiP transiently associated with many proteins in the wildtype mouse embryonic fibroblasts (Fig. 4-7). The majority of these substrates were of a relatively high molecular weight reminiscent of the calreticulin specific substrates seen in the Figure 4-6. It was interesting to note, however, that there was a significant increase in the quantity of protein associated with BiP in calreticulin deficient cells (Fig. 4-7, *crt*$^{-/-}$). Not only did a greater amount of protein associate with BiP in the calreticulin deficient cells, but also they remained associated for a greater period of time as seen by the proteins chasing off at later times (up to 45 min as compared to 20 min in wildtype). These findings suggested that calreticulin specific substrates formed complexes with BiP. To test if the inhibition of the interaction of newly synthesized glycoproteins with calnexin and calreticulin was sufficient to drive these to associate with BiP, we used the glucosidase inhibitor

castanospermine. As seen previously, treatment of the cells with castanospermine resulted in a dramatic loss of substrate binding to calnexin (Fig. 4-4). When labelled cell lysates from castanospermine treated cells were used to immunoprecipitate BiP, we observed that an increased amount of proteins coprecipitated with BiP, which was most noticeable in the wildtype cells (Fig. 4-8), as compared to when the cells were left untreated (Fig. 4-7). From these results we concluded that in the absence of binding to calreticulin, substrate proteins were picked up by a chaperone outside of the calreticulin/calnexin cycle, namely BiP.

Conclusions and Discussion

This part of the study focused on the function of ER chaperones. We studied the effect of the absence of calreticulin on the chaperoning system of the ER. We first observed that without calreticulin, the function of calnexin was impaired. Calnexin was unable to bind to its substrate proteins for the normal period of time, and these substrates dissociated from calnexin at a faster rate. We also saw that under conditions where glycan binding to calnexin was inhibited, the direct peptide binding function of calnexin was also impaired in calreticulin deficient cells. By using the proteasome inhibitor ALLN, we saw an increased association of substrate proteins with calnexin at later time points in the calreticulin deficient cells. We concluded from this that in the absence of

calreticulin, the function of calnexin was impaired and this resulted in an accumulation of unfolded proteins. We observed the most interesting finding when we looked at the function of BiP, an ER chaperone that is not involved in the calnexin/calreticulin cycle. The results from the BiP immunoprecipitation showed that there was increased substrate association in the calreticulin deficient cells. Also when substrate binding to calreticulin/calnexin was inhibited using castanospermine, this increase in association with BiP was also observed. From these results it is apparent that BiP will pick up substrates of calreticulin if binding to calreticulin is prevented, and it seems that BiP functions as a back up or compensates for the loss of calreticulin in the knockout cells. Overall, the majority of calreticulin substrates were handed over to BiP or accumulated as unfolded proteins in the ER lumen. These results provide insight into the ER protein folding pathway and the function of the chaperones in this system, and indicate that in the absence of calreticulin quality control in the ER is compromised.

Figure 4-1. Immunoprecipitation of calnexin from wildtype and calreticulin deficient cells. Cells were pulse-labelled with [^{35}S] methionine/cysteine and lysates used for immunoprecipitation with anti-calnexin antibodies as described in Materials and Methods. Shown is the phosphorimager image of the immunoprecipitates on the dried gel after SDS-PAGE on 8% acrylamide gels. *crt*$^{-/-}$ indicates calreticulin deficient cells, and *crt*$^{-/-}$ + rCRT are calreticulin deficient cells backtransfected with recombinant calreticulin. The protein bands indicated by • are those in which the kinetic difference between wildtype and calreticulin deficient cells is clearly visible and are discussed in the text.

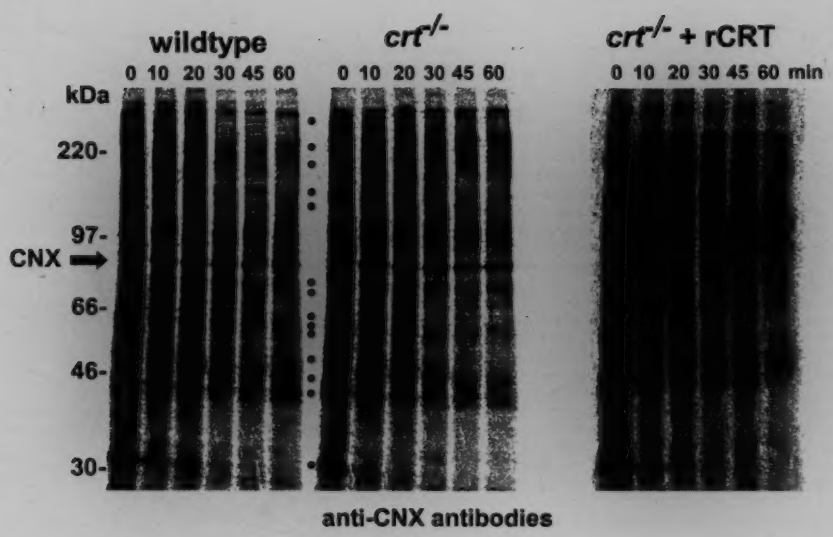

wildtype
crt-/-
crt-/- + rCRT
0 10 20 30 45 60
0 10 20 30 45 60
0 10 20 30 45 60 min
kDa
220-
97-
CNX
66-
46-
30-
anti-CNX antibodies

Figure 4-1

Figure 4-2. Quantitation of protein bands associated with calnexin in immunoprecipitates from wildtype and calreticulin deficient cells. Wildtype and calreticulin deficient cells were pulse-labelled and lysates used for immunoprecipitation with anti-calnexin antibodies as in Figure 4-1. Shown is the phosphorimager image of the immunoprecipitates on the dried gel after SDS-PAGE on 8% acrylamide gels. The protein bands at the indicated molecular weights were quantitated and each plotted as arbitrary units for both cell types.

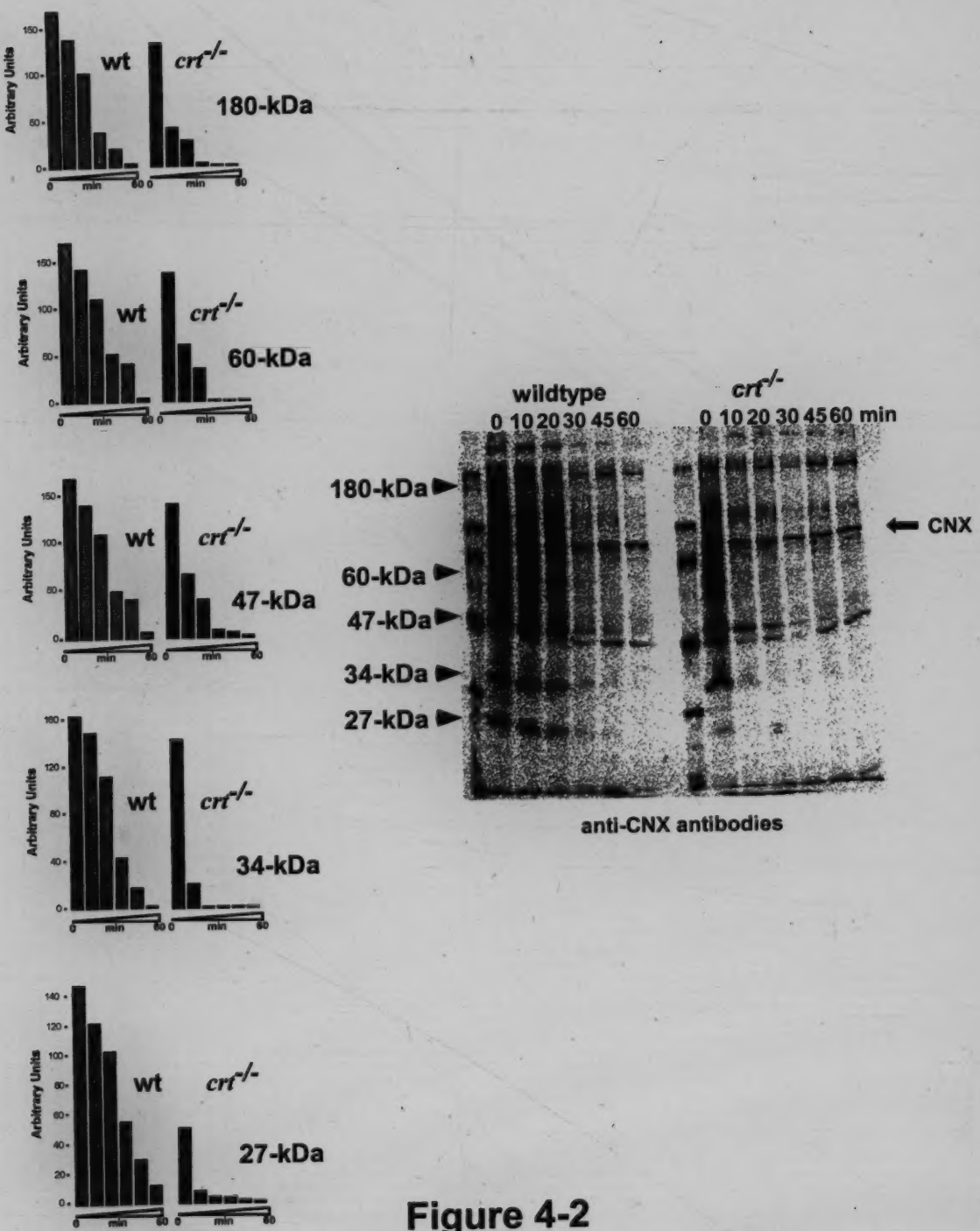

Arbitrary Units
wt
crt-/-
180-kDa
60-kDa
47-kDa
34-kDa
27-kDa
min
wildtype
0 10 20 30 45 60
crt-/-
0 10 20 30 45 60 min
CNX
anti-CNX antibodies

Figure 4-2

Figure 4-3. Immunoprecipitation of calnexin from wildtype and calreticulin deficient cells after tunicamycin treatment. Cells were treated throughout the entire experiment with 20 μg/mL tunicamycin before immunoprecipitation as described in Materials and Methods. Shown is the phosphorimager image of the immunoprecipitates on the dried gel after SDS-PAGE on 8% acrylamide gels. Open arrow indicates protein band in which kinetic differences are observed between the two cell types and is discussed in the text.

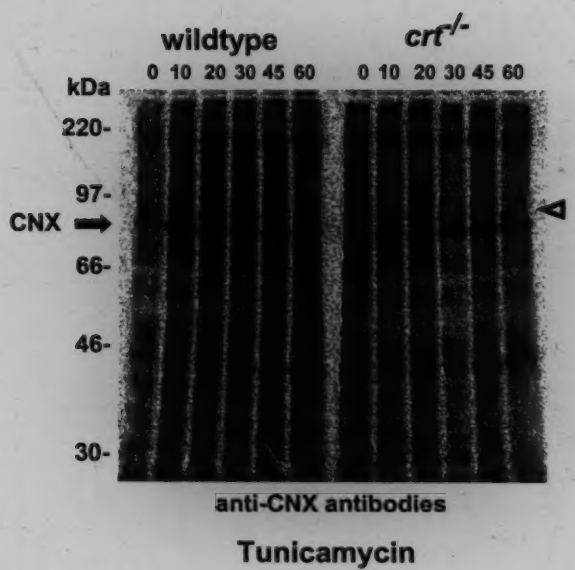

wildtype
crt-/-
0 10 20 30 45 60
0 10 20 30 45 60
kDa
220-
97-
CNX
66-
46-
30-
anti-CNX antibodies
Tunicamycin

Figure 4-3

Figure 4-4. Immunoprecipitation of calnexin from wildtype and calreticulin deficient cells after castanospermine treatment. Cells were treated throughout the entire experiment with 0.5 mM castanospermine before immunoprecipitation as described in Materials and Methods. Shown is the phosphorimager image of the immunoprecipitates on the dried gel after SDS-PAGE on 8% acrylamide gels. Open arrow indicates protein band in which kinetic differences are observed between the two cell types and is discussed in the text.

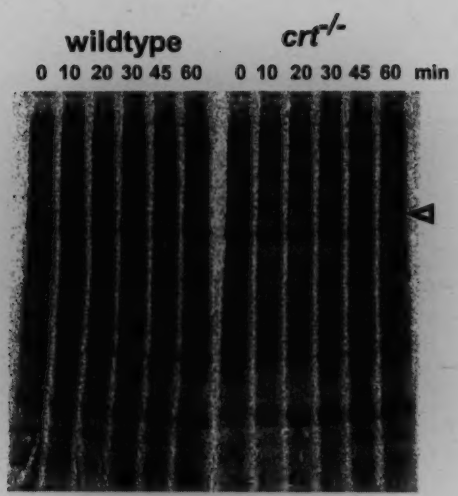

wildtype
crt-/-
0 10 20 30 45 60
0 10 20 30 45 60 min
anti-CNX antibodies
Castanospermine

Figure 4-4

Figure 4-5. Immunoprecipitation of calnexin from wildtype and calreticulin deficient cells after ALLN treatment. Cells were treated throughout the entire experiment with 50 μg/mL ALLN before immunoprecipitation as described in Materials and Methods. Shown is the phosphorimager image of the immunoprecipitates on the dried gel after SDS-PAGE on 8% acrylamide gels. Arrows indicate protein bands where an accumulation of protein is observed in the calreticulin deficient cells. These bands are discussed in the text. ALLN, N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal.

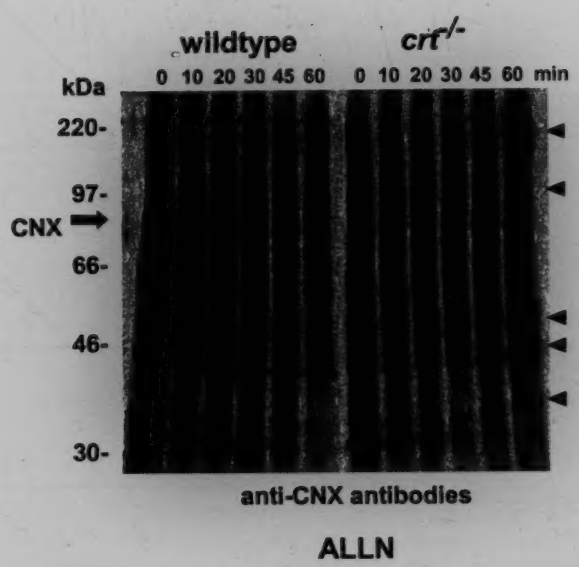

wildtype
crt-/-
0 10 20 30 45 60 0 10 20 30 45 60 min
kDa
220-
97-
CNX
66-
46-
30-
anti-CNX antibodies
ALLN

Figure 4-5

Figure 4-6. Immunoprecipitation of calreticulin from wildtype and calreticulin deficient cells. Wildtype and calreticulin deficient cells were pulse-labelled and lysates used for immunoprecipitation with anti-calreticulin antibodies. Shown is the phosphorimager image of the immunoprecipitates on the dried gel after SDS-PAGE on 8% acrylamide gels. The calreticulin deficient cells were used as a control for this antibody. Those bands observed from these cells are non-specific.

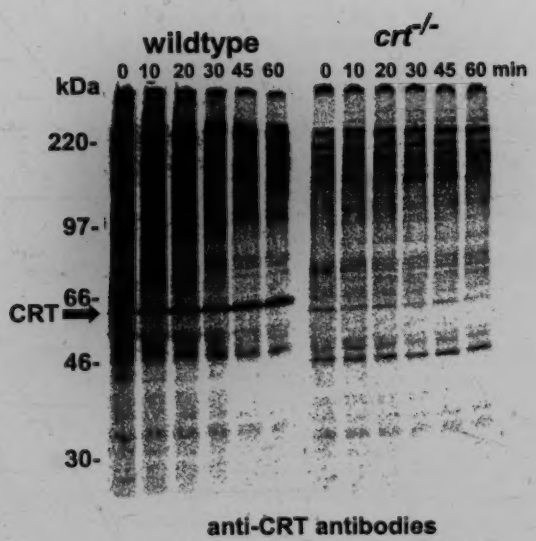

wildtype
crt-/-
0 10 20 30 45 60 0 10 20 30 45 60 min
kDa
220-
97-
66-
CRT
46-
30-
anti-CRT antibodies

Figure 4-6

Figure 4-7. Immunoprecipitation of BiP from wildtype and calreticulin deficient cells. Wildtype and calreticulin deficient cells were pulse-labelled and lysates used for immunoprecipitation with anti-BiP antibodies as described in Materials and Methods. Shown is the phosphorimager image of the immunoprecipitates on the dried gel after SDS-PAGE on 8% acrylamide gels.

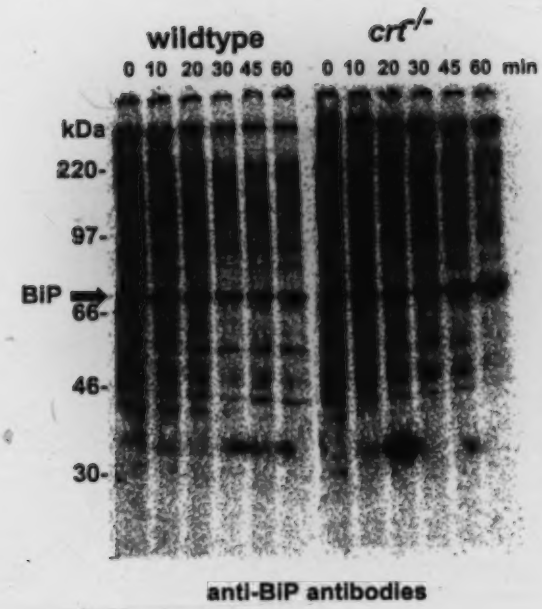

wildtype
crt-/-
0 10 20 30 45 60 0 10 20 30 45 60 min
kDa
220-
97-
BiP
66-
46-
30-
anti-BiP antibodies

Figure 4-7

Figure 4-8. Immunoprecipitation of BiP from wildtype and calreticulin deficient cells after castanospermine treatment. Cells were treated throughout the entire experiment with 0.5 mM castanospermine before immunoprecipitation as described in Materials and Methods. Shown is the phosphorimager image of the immunoprecipitates on the dried gel after SDS-PAGE on 8% acrylamide gels.

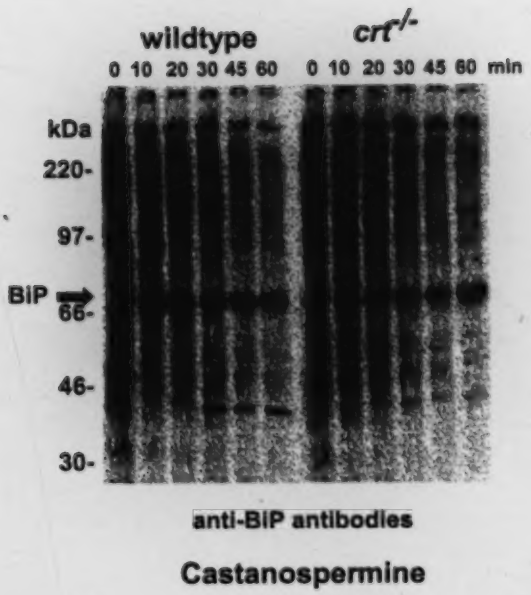

wildtype
crt-/-
0 10 20 30 45 60 0 10 20 30 45 60 min
kDa
220-
97-
BiP
66-
46-
30-
anti-BiP antibodies
Castanospermine

Figure 4-8

GENERAL SUMMARY AND DISCUSSION

In this study, we used cells deficient in the ER Ca^{2+} binding chaperone calreticulin to study the pathway of protein folding and the chaperone system in the endoplasmic reticulum (ER). We first looked at the effect of the absence of calreticulin on the expression profile of various ER proteins and chaperones which assist in the folding of newly synthesized polypeptides. Western blot analysis of cells from wildtype and calreticulin deficient mice showed that there was a significant change in the profile of ER chaperones in the absence of calreticulin. Specifically, calnexin and the α and β subunits of glucosidase II were upregulated. Although there was a significant decrease in the amount of PDI in the calreticulin deficient cells, the thiol oxidoreductases ERp57 and ERp72 were upregulated in these cells. At this point, it is difficult to explain the downregulation specifically of PDI in the absence of calreticulin. Although they are all part of the same family of proteins (thiol oxidoreductases), further studies on the differences between the individual functions of PDI, ERp57, and ERp72 will perhaps help explain this observation. There were also increases in the general chaperones BiP and Grp94. This trend of upregulated chaperones in the calreticulin deficient cells was quite interesting. First these results imply that in the absence of an ER chaperone, other chaperones are upregulated to compensate for the loss of its function. Since BiP and Grp94 were also significantly upregulated (see Table 3-1), this suggests that the lectin cycle may not function correctly without calreticulin and other chaperone systems are therefore required to compensate. The results observed showing increases in ER chaperones also suggest that the unfolded protein response pathway is activated in the calreticulin

deficient cells, as an upregulation of ER chaperones and in particular increased BiP is a general marker for an activated UPR pathway (Gething, 1999; Kaufman, 1999; Mori, 2000). We observed that both Ire1 and PERK, both proteins which transduce the ER stress signal to the nucleus (Tirasophon et al., 1998), were both upregulated in the calreticulin deficient cells. These findings indicated that indeed the UPR pathway was activated in the calreticulin knockout cells. Since BiP synthesis increases during conditions that lead to the accumulation of unfolded polypeptides in the ER (Gething, 1999), and we observed this phenomenon in the calreticulin deficient cells, this suggests that the accumulation of unfolded proteins may be the result of the loss of calreticulin. This indicates that calreticulin plays a vital role as a chaperone and that the degree of redundancy among chaperones is lower than previously thought since there are deficiencies in the folding pathway when one chaperone is absent.

The next section of the study focused on the function of ER chaperones in the absence of calreticulin. The loss of calreticulin impaired the function of calnexin. Calnexin was unable to bind to its substrate proteins for the normal period of time since they dissociated from calnexin at a faster rate. Since the role of chaperones is to bind and retain unfolded proteins until they are completely folded (Helenius et al., 1997; Stevens and Argon, 1999), this finding may suggest that protein trafficking was accelerated in the calreticulin deficient cells, and their transit time in the ER lumen was decreased. This may also indicate that calnexin and calreticulin function by slowing down the flow of proteins through the

secretory pathway and function by retaining their substrates in the ER for a period of time to allow folding to occur. In cells treated with the N-glycosylation inhibitor tunicamycin, substrate binding to calnexin was almost completely abolished, which complies with calnexin's specificity for monoglucosylated glycoproteins (Helenius et al., 1997). By using the proteasome inhibitor ALLN, we saw an increased association of substrate proteins with calnexin at later time points in the calreticulin knockout cells. We concluded from this that in the absence of calreticulin, the function of calnexin was impaired and this resulted in an accumulation of unfolded proteins. Comparing immunoprecipitations from both calnexin and calreticulin antibodies suggested that calnexin and calreticulin had quite a different set of chaperone substrates. This was rather unexpected considering the lectin specificities of both calnexin and calreticulin are identical, thus an extensive overlap between their substrate glycoproteins should occur. This difference suggests that the presence of a glycan moiety may not be the only requirement for interaction of calnexin and calreticulin and other factors may be involved, such as protein-protein interactions or perhaps the position of the glycan chain on the peptide. Also, the observation that few substrates were shared between calnexin and calreticulin in this study, might help to explain why the calreticulin knock out mouse is embryonic lethal (Mesaeli et al., 1999) since calnexin would be unable to substitute for calreticulin.

We obtained an insightful observation when we looked at the function of BiP, an ER chaperone that is not involved in the calnexin/calreticulin cycle. The

results from the BiP immunoprecipitation showed that there was increased substrate association in the calreticulin deficient cells. Also when substrate binding to calreticulin/calnexin was inhibited using castanospermine, this increase in association with BiP was again observed. From these results it is apparent that BiP will pick up substrates of calreticulin if binding to calreticulin is prevented, and it seems that BiP functions as a back up or compensates for the loss of calreticulin in the knockout cells. Overall, the majority of unfolded calreticulin substrates were handed over to BiP or accumulated as unfolded proteins in the ER lumen. These results provide an insight into the ER protein folding pathway and the function of the chaperones in this system. We observed from this study that ER chaperones were not entirely redundant and although the expression patterns of ER chaperones suggest that there is a compensatory action occurring when calreticulin is not present, that fact the calnexin's function is compromised suggests that this action is insufficient, and that although other chaperones such as BiP played a role in somewhat compensating for calreticulin, this was not completely sufficient to replace the function of calreticulin.

The most important finding of this study is that the absence of one ER chaperone is sufficient to alter the overall folding of nascent glycoproteins and to distort the intralumenal equilibrium in the ER, resulting in a compromised quality control pathway. A general model of the findings of this study is shown in Figure 5-1. Under normal conditions there may be two distinct chaperone systems in the ER, one comprising BiP, PDI and Grp94, the other ERp57, calnexin, and

calreticulin (Molinari and Helenius, 2000) (Fig. 5-1, upper panel). However, it is likely that some overlap between the two systems exists, since the absence of one chaperone (calreticulin deficiency) impacts the folding process in both pathways. In calreticulin deficient cells, the association between calnexin and its substrates is compromised, leading to accumulation of unfolded proteins and glycoproteins (Fig. 5-1, lower panel). This indicates that calreticulin is essential for the normal function of calnexin as a chaperone. In the absence of calreticulin, calreticulin substrates are not picked up by calnexin, but become associated with BiP, accumulated as unfolded proteins, or secreted (Fig, 5-1, lower panel). BiP, PDI, ERp57 and Grp94 function is also impaired in calreticulin deficient cells, leading to further accumulation of unfolded proteins (Fig, 5-1, lower panel). The expression of Ire1 and PERK kinases is induced in calreticulin deficient cells, indicating that calreticulin dependent disruption of quality control processes leads to activation of the unfolded protein response, and to increased expression of the ER stress proteins BiP and Grp94 (Fig, 5-1, lower panel). The increase in unfolded proteins and induction of the UPR pathway seen in calreticulin deficient cells demonstrate that calreticulin is essential in the normal quality control processes associated with ER chaperone function.

The mechanisms responsible for calreticulin dependent changes in the ER chaperone function may be many. It is known that ER lumenal chaperones, including calreticulin, form functional complexes (Koch, 1987; Tatu and Helenius, 1997), which are easily broken in the absence of one of the chaperone

component. These may rely on Ca^{2+}-dependent interactions between calreticulin and other ER associated chaperones (Corbett et al., 2000; Koch, 1987), and Ca^{2+} has been shown to be a factor in directly regulating the association between calreticulin, PDI, and ERp57 (Corbett et al., 1999). Perturbation of Ca^{2+} homeostasis may cause the disruption of these ER chaperone complexes leading to impaired chaperone function and an accumulation of unfolded proteins, which in turn may underlie the lethality associated with calreticulin-deficiency. This interrelationship between Ca^{2+} and chaperoning is further supported in that many ER chaperones are Ca^{2+} binding proteins and that their lumenal interactions and activities are regulated in a Ca^{2+}-dependent fashion (Corbett et al., 1999). Therefore, the absence of calreticulin may also affect the function of other chaperones and folding enzymes whose activities have been demonstrated to be dependent on Ca^{2+} homeostasis such as ERp57 (Oliver et al., 1999), BiP (Lievremont et al., 1997) and PDI (Baksh et al., 1995; Corbett et al., 1999).

The major observations and conclusions made from this study were that, in the absence of calreticulin, the function of ER chaperone proteins are compromised. However, these observations may only be a consequence of secondary effects induced by the absence of calreticulin. Calreticulin is a major Ca^{2+} binding protein in the ER and plays a major role in cellular Ca^{2+} homeostasis (Corbett and Michalak, 2000; Michalak et al., 1999). Although the loss of calreticulin from cells does not affect the free ER Ca^{2+} concentration, there is an approximately two-fold decrease in the total ER Ca^{2+} content in calreticulin

deficient cells (Nakamura et al., 2001). This rather significant change in ER Ca^{2+} content might adversely affect the functions of ER proteins that are dependent on Ca^{2+}. For example, for both calreticulin and calnexin, Ca^{2+} is essential for their lectin-like behaviour. *In vitro* experiments showed that both these proteins cannot bind free oligosaccharide in the complete absence of Ca^{2+} (Vassilakos et al., 1998). This might be a reason why, in this study, decreased substrate binding to calnexin was observed in the calreticulin deficient cells. In addition, many other components of the ER chaperone system are dependent on intracellular Ca^{2+} concentration such as PDI and ERp57 (Corbett et al., 1999). Also, Ca^{2+} in general is an important signalling molecule and plays an essential role in many cellular activities (Berridge et al., 2000), and therefore a change in intracellular Ca^{2+} concentration would affect the cell's function in many ways, including the activities of ER chaperones. Although all these Ca^{2+} dependent effects are a possibility, the actual free ER Ca^{2+} concentration remains unchanged in calreticulin deficient cells (Nakamura et al., 2001), and the data showing the upregulation of various ER chaperone proteins indicate that there are other factors at work, and therefore the results observed must be somewhat specific to the chaperone function of calreticulin. There are, nevertheless, increasing reports of disease states associated with ER protein folding and retention of unfolded proteins. This work shows that susceptibility to conformational diseases, such as Alzheimer's disease, familial hypercholesterolemia, and cystic fibrosis may arise if the function of a certain ER chaperone is compromised. It also highlights the essential role of calreticulin for quality control in the ER of mammalian cells.

The challenge for the future is to define the roles that chaperones play in the folding of proteins and identify the specific interactions between the chaperone and substrate that aid this process. With the recent identification of the crystal structure of calnexin (Schrag et al., 2001), and the NMR structure of the P-domain of calreticulin (Ellgaard et al., 2001), progress is already being made in determining how calreticulin and calnexin may interact with their substrates and other chaperone proteins (Ellgaard and Helenius, 2001). The specific role that N-linked glycans play in these interactions would also be important to identify. Identifying different pathways for folding substrates and determining whether these are specific for certain proteins will also provide further insight into the quality control process. Determining the specific mechanism that protein chaperones use in the folding process will to help build strategies to manipulate and control protein folding in the cell. In the future, this ability to modulate protein folding and quality control in the ER may prove essential in the treatment of conformational diseases with etiologies stemming from defective protein folding.

Figure 5-1. General model showing the ER protein folding pathway in calreticulin deficient cells. Under normal conditions there may be two distinct chaperone systems in the ER, one comprising BiP, PDI and Grp94, the other ERp57, calnexin, and calreticulin (upper panel). Newly synthesized proteins normally associate with various chaperones in the ER and then are released when folding is completed. In calreticulin deficient cells, the association between calnexin and its substrates is compromised, leading to accumulation of unfolded proteins and glycoproteins (lower panel). In the absence of calreticulin, the majority of its substrates are directed to BiP, accumulated as unfolded proteins, or secreted (lower panel). The function of other chaperones may also be impaired in calreticulin deficient cells, leading to further accumulation of unfolded proteins (lower panel). The expression of Ire1 and PERK kinases is induced in calreticulin deficient cells, indicating that calreticulin dependent disruption of quality control processes leads to activation of the unfolded protein response, and to increased expression of the ER stress proteins BiP and Grp94 (lower panel).

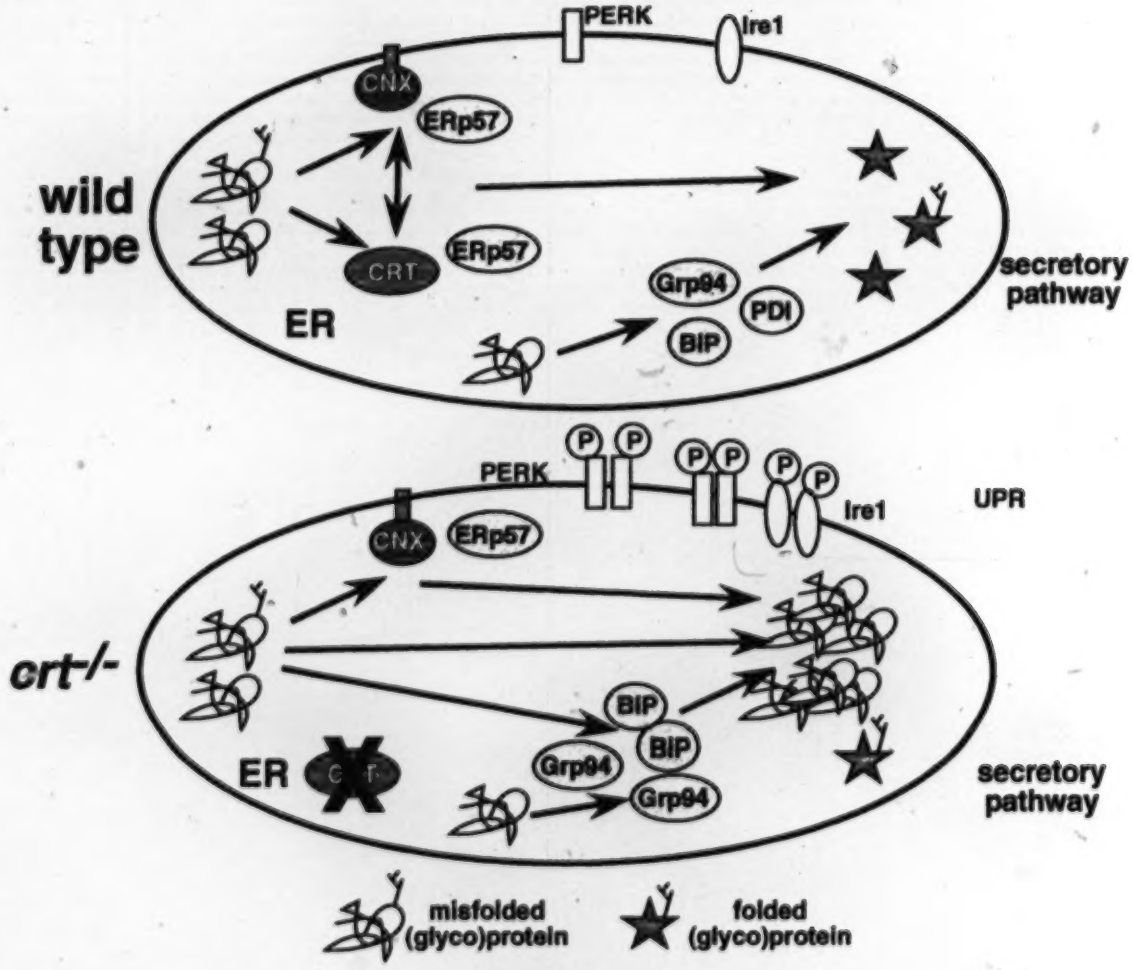

PERK
Ire1
CNX
ERp57
wild type
CRT
ERp57
ER
Grp94
PDI
BiP
secretory pathway
P
PERK
Ire1
UPR
CNX
ERp57
crt-/-
BiP
BiP
ER
Grp94
Grp94
secretory pathway
misfolded (glyco)protein
folded (glyco)protein

Figure 5-1

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